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Remyelination Biology

The Neurobiology of Oligodendrocyte Progenitor Cells and Their Potential for Myelin Repair in Multiple Sclerosis

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Abstract

Oligodendrocyte damage and myelin loss are cardinal features of Multiple Sclerosis (MS). Intrinsic myelin repair occurs in MS, mediated by quiescent oligodendrocyte progenitors that divide and migrate into demyelinated lesions. Experimental remyelination suggests that this repair restores function and can protect axons from subsequent degeneration. However this repair is limited, and disability supervenes. Designing treatments that augment myelin repair is both feasible and attractive.

Much is known about the rodent oligodendrocyte lineage, but significant species differences exist and extrapolation to humans requires direct experimental support. Human oligodendrocyte progenitors are hard to grow *in vitro*, and supplies of source tissue and cellular yield are both limited. This problem is exacerbated by the failure of rodent mitogens to induce equivalent growth expansion of human progenitors. Several possible methods could be employed to circumvent these difficulties:-

A conditionally immortalised human progenitor cell line transfected with a temperature sensitive oncogene has been reported. However, it was demonstrated that all stocks of this cell line have been irredeemably contaminated with rodent cells.

It has been suggested that rodent progenitors can dedifferentiate into a more proliferative, multipotent phenotype. If dedifferentiation was a feasible method of inducing committed progenitors to a more proliferative state, it might be exoected that this property would be widespread amongst similar cells. The rodent progenitor cell line CG4 did not dedifferentiate in these circumstances, although the original experiment was not repeated. This exemplifies the problems of assessing lineage commitment using cell lines, while attesting to the stability of CG4.

Primary cultures of glia from surgical specimens can yield small numbers of oligodendrocyte progenitors. Identifying these cells traditionally relies on the

morphology and the expression of A2B5 antigens. Some studies have used NG2, an established marker of developing rodent progenitors but there is little experimental evidence to support its use in adult humans. It was shown that this antibody binds human endothelial cells, fibroblasts and certain types of astrocytes and thus lacks specificity for the oligodendrocyte lineage *in vitro*, although the proportion of cells staining with these markers requires further study. A population of bipolar and clawed cells, unidentified by traditional markers, appears to label with NG2.

Purification of oligodendrocyte lineage cells using magnetic beads was optimised and there was preliminary evidence that the resulting cells proliferate *in vitro*. They gave rise to a population of small bipolar cells that were did not express A2B5 antigens but stained for NG2. We believe these to be of the oligodendrocyte lineage and further investigation of these cells is required.

The emergent reports of stem cells in the adult mammalian brain were supported by studies using adult human tissue. These cells grow in aggregate cultures and can be induced to express oligodendrocyte markers. The feasibility of this approach as a source of oligodendrocyte lineage cells will rely on further work to ensure that their progeny remain faithful to native oligodendrocytes.

Finally, rodent cells were used to establish that two key determinants of myelinating cell efficiency, migration and proliferation, are resistant to the effects anti-inflammatory drugs used in MS. It is anticipated that this type of research will soon be possible using human cells.

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Dedication and Acknowledgements

I would like to dedicate this to my beloved wife Sue and to my wonderful children Will and Emmie. They have supported me with love, encouraged me to persevere and have born my absence with patience. They have refreshed me with their fun, and continue to give me inexpressible joy. Above all they have reminded me that the Lord is sovereign over all things, He created the biology that I have been investigating, and it is only through His strength that I have been able to do this work. May all that I have achieved be for His greater glory!

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Finally I would like to thank my parents, parents-in-law, family and friends for their love, support, encouragement, humour and wisdom.

AUTHOR'S DECLARATION

I declare that the work in this dissertation was carried out in accordance with the Regulations of the University of Bristol. The work is original except where indicated by special reference in the text and no part of the dissertation has been submitted for any other degree. Chapter 6 has been published (Halfpenny & Scolding 2003) and was co-authored by Prof. N.J.Scolding who supervised this work, and some of the text has been reproduced from tracts written predominantly by the author, but published with co-authors Prof N.J.Scolding and Dr T.M.Benn (Halfpenny et al. 2002). It is reproduced here with some minor changes. Any views expressed in the dissertation are those of the author and in no way represent those of the University of Bristol. The dissertation has not been presented to any other University for examination either in the United Kingdom or overseas.

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Chapter 1. General Introduction

Multiple Sclerosis

Multiple Sclerosis (MS) is the commonest progressive neurological condition affecting young people in Britain. It affects some 55,000 people in this country alone. It has devastating consequences to the patients, and far-reaching social and economic sequelae. Despite much publicity, the treatments available are disappointing, expensive and ultimately fail to reverse or halt the disease.

The disease is typified by episodes of discrete neurological deficits progressing over days and resolving, often completely, over weeks. They characteristically affect different parts of the central nervous system (CNS) at different times, but certain regions appear particularly susceptible. Common manifestations include unilateral visual loss from optic neuritis, ataxia and diplopia from brainstem lesions and sensorimotor symptoms from spinal cord involvement. These neurological episodes are irregular, but typically occur slightly less than once a year (Compston & McAlpine 1998) (relapsing-remitting phase).

However, with time, previously dramatic recovery from attacks becomes less complete, and disability accumulates (secondary progressive phase). Relapses may become less frequent or cease altogether. Spasticity, disabling ataxia and sphincter disturbance are common accompaniments of chronic disease. A proportion of patients (30%) develop progressive disease from the outset, and one-third of these never manifest acute relapses (Weinshenker et al. 1989) (primary progressive multiple sclerosis).

The majority of research into MS has been aimed, directly or indirectly, at finding ways of controlling disease activity, and hence preventing progression. Though self-evidently wholly necessary, this approach ignores the disability already suffered by many.

Aetiology and pathology

Despite a century and a half's interest and enormous investment in MS research, no causal hypothesis has been universally accepted. Controversies abound, as is amply demonstrated by the extraordinarily diverse hypotheses and counter-hypotheses currently proposed (Behan et al. 2002;Compston & McAlpine 1998;Hawkes 2002).

While many aspects of the disease do remain frustratingly elusive, painstaking research has provided a broadly coherent picture of disease pathogenesis, which requires a brief summary.

Important evidence comes from epidemiological studies. These reveal a complex interplay between genetic susceptibility and environmental triggers. The genetic contribution has been systematically explored; some 5-7 regions of the human genome are consistently identified, but none of these appear to carry alleles that are either sufficient or necessary for development of the disease (Compston 1999). However, consistent associations with the regions encoding the HLA complex and cytokine (TNFα) genes are important evidence for an immunological component to disease aetiology. The unusual epidemiology of the disease, and in particular the marked geographical variation and existence of restricted outbreaks (eg Faeroe Islands), implicate environmental agents, of which viruses have always been prime contenders. However, while a clear relationship between relapse and viral infection has been established (Sibley et al. 1985), and many viruses have been proposed as contenders, no viral agent has ever been convincingly demonstrated as a cause for the disease.

Further important clues come from detailed pathological studies. Charcot described the essential pathological features of MS in the nineteenth century (Charcot 1868), and his observations have been confirmed and extended several times since. The classical pathological hallmarks of the disease are of areas of inflammation (plaques), often localised around blood vessels, in the brain and spinal cord. These contain prominent myelin destruction and oligodendrocyte death, with relative, but not absolute, preservation of axons. More recently it has been recognised that there are widespread abnormalities, including diffuse inflammation, small foci of demyelination (Allen & McKeown 1979) and axonal changes (Trapp et al. 1998) in macroscopically normal appearing white and grey matter as well.

While these three cardinal features of MS; inflammation, myelin loss, and relative axonal sparing, are present in all patients, there are sufficient differences between individuals to suggest that the pathogenic mechanisms may not be entirely uniform between MS patients. A study of active demyelinating lesions in a large series of MS patients found evidence for four different patterns of pathological findings that were conserved within lesions from the same patient. The most common pattern (designated pattern II), found in just over half of these patients, suggested myelin damage

consequent on both T cell mediated destruction and antibody deposition. A related, but rarer pattern (I) was similar but lacked notable antibody mediated destruction. In both of these, myelin loss was centred on veins, providing further support for an inflammatory cause for the myelin loss. These patterns are very similar to those seen in experimental allergic encephalomyelitis (EAE) which has been widely used as a model for MS. However the second commonest pattern of pathology (III), seen in about 30% of the patients, was unlike that seen in any of the EAE models. The most notable feature in biopsies and autopsies from these patients was premature loss of myelin associated glycoprotein (MAG) immunoreactivity. MAG is expressed by the periaxonal, inner myelin lamellae and it is consequently at the furthest (and most metabolically vulnerable) extremity of the oligodendrocyte. Thus early loss of this protein implies selective damage to this metabolically vulnerable region, and hence suggests primary damage to the oligodendrocyte itself. The demonstration of apoptotic oligodendrocytes and prominent loss of oligodendrocyte numbers in the demyelinated plaque centre supports this. While inflammatory changes were seen in these lesions, the periaxonal part of the myelin sheath is relatively protected from immune attack, and the demonstration of relative myelin preservation around venules also tends to exonerate inflammation as the primary mechanism of myelin injury in these patients. This pattern of pathology is also seen in diseases where oligodendrocyte damage is caused by viruses or toxins. The rarest pattern (IV), found only in primary progressive MS patients, also showed prominent oligodendrocyte loss but without premature loss of MAG or evidence for apoptosis within the lesions (Lucchinetti et al. 2000).

These variations may have a number of origins. Variability in the effecter mechanisms of an immune response between individuals with a different genetic background is seen in EAE, but pattern III is particularly difficult to explain with a pure immune hypothesis. The authors of the report suggest that this variability might reflect different stages in the disease or even fundamental difference in the pathogenesis between different patients. There are insufficient data presently available to differentiate between these possibilities with any certainty. This study does, however, re-emphasise the limitations of EAE as a model for MS a whole.

The sequence of events that lead to this triad of inflammation, myelin loss and axonal damage remain to be fully elucidated. Disruption of the blood brain barrier has traditionally been one of the earliest identified pathological events in lesion formation (Compston & McAlpine 1998) and the first evidence of the inflammatory phase. This

can be demonstrated by gadolinium enhancement on MRI, often mirroring the development of clinical symptoms (Miller & Thompson 1999). In that the blood-brain barrier usually restricts the exposure of intrinsic brain antigens to systemic immune surveillance, damage to this is thought to lead to antigen recognition and hence to an escalating immune response. Activation of infiltrating macrophages and resident microglia, release of pro-inflammatory cytokines and chemokines, up-regulation of their receptors, increased expression of adhesion molecules, MHC antigens and a range of co-stimulatory molecules (Lassmann 1999) have all been demonstrated. These findings all appear to support a primarily immune hypothesis of MS pathogenesis, similar to that seen in EAE, and consistent with the pathology in patterns I and II.

During the period of active inflammation, a process of myelin destruction occurs, but there are several pointers to suggest that these processes may be separate. It is notable that many of the inflammatory features of MS are seen in other CNS diseases, but the degree of demyelination is unique to MS (Lassmann 1999). A major demyelinating mechanism in many patients appears to be antibody deposition and subsequent complement activation on myelin sheaths (pattern II), which is demonstrable at the site of active myelin destruction (Storch et al. 1998). This has similarities with a widely used EAE model; myelin oligodendrocyte glycoprotein (MOG)-induced EAE in Lewis rats. In this particular model, inflammatory lesions can be induced by transfer of myelin basic protein (MBP)-reactive T-cells, but little demyelination occurs unless antibodies directed against MOG are injected at the same time as the T-cells. Antibody and complement deposition is not a universal finding in MS (Lucchinetti et al. 2000) and although many patients (but not all) have antibodies to MOG detectable in their CSF, these can also be found in patients with other CNS inflammation (Reindl et al. 1999) and so cannot be sufficient for demyelination alone.

Other mechanisms for demyelination may co-exist, or dominate the pathology in some patients. The oligodendrocyte itself, rather than the myelin sheath, may be the primary target of attack (patterns III & IV). The high metabolic load required to maintain the myelin sheath makes the oligodendrocyte very vulnerable to injury, and direct oligodendrocyte cytotoxicity from a range of agents has been demonstrated, many of which have been shown to occur in MS lesions. These include nitric oxide and its highly reactive metabolite peroxynitrite, perforins and the cytokines TNFα and IFNγ

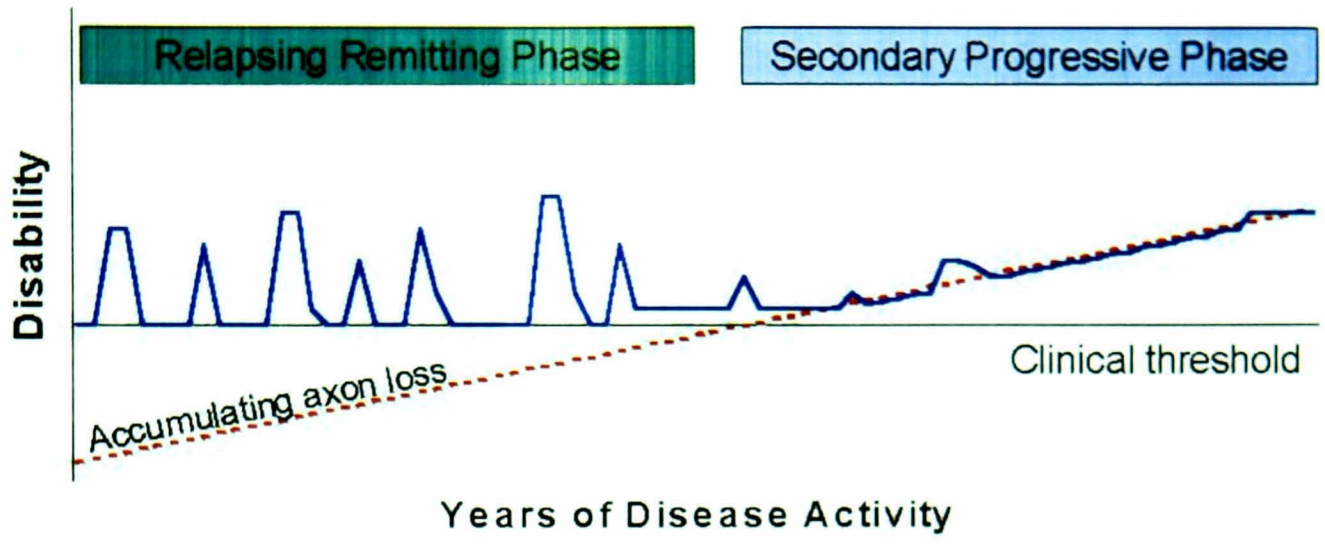
(Benn et al. 2001). The presence of apoptosis and the up-regulation of molecules involved in death-related signalling have also been demonstrated in MS lesions, but the significance of these remain open to debate (Rodriguez & Lucchinetti 1999). Recent insights from more advanced MRI sequences have challenged the traditional view that myelin and axonal damage follow damage to the blood brain barrier. Magnetisation transfer ratios, which are particularly sensitive to pathological changes in myelin and axons, decrease focally months or even years before lesions become visible with T_2-weighted (Goodkin et al. 1998;Pike et al. 2000) or gadolinium enhanced (Filippi et al. 1998) images. The causes for these early changes are unclear, but they have important implications for our understanding of lesion development and challenge the rational of some current therapies. These findings raise the intriguing possibility that inflammation and plaque formation may be the culmination of a longstanding pathological process, rather than its inception. If confirmed, the role of inflammation as the prime mechanism of damage is in doubt, indeed it may play a much more benign role (discussed further in Chapter 6).

The final component of the triad, axonal damage, was reported in the earliest descriptions of MS, but it is the recent attempts to quantify it that have fuelled a resurgence of interest in this component of the disease. It is worth re-emphasising that axons are relatively spared, particularly in comparison with the widespread myelin loss. However, the traditional histopathological features of axonal swellings and axon bulb formation proximal to the site of axon damage and fragmentation were described in 1936 (Greenfield & King 1936). These are specific for axonal damage – they occur in axonal dystrophies, be they primary or secondary, but not in neurodegenerative conditions where the neuronal soma is primarily targeted. However they lack sensitivity, which limited these few early attempts to quantify axonal damage in MS (Greenfield & King 1936). Recent studies have been assisted by newer and more sensitive techniques. One of these includes the immunohistochemical demonstration of β amyloid precursor protein (APP). This is normally carried down the axon by fast axonal transport, but it accumulates immediately proximal to foci of axonal injury where it can be demonstrated by immunohistochemical methods. The proliferation of new techniques of magnetic resonance based imaging has allowed estimation of axon loss in living patients, and this has enabled longitudinal clinical studies to be performed. These studies use quantitative MR spectroscopy to determine levels of N-

acetyl-aspartate (NAA). Since this substance is mostly expressed in axons (Bjartmar et al. 2002) (although some rodent studies suggest that oligodendrocyte progenitors may also express it (Bhakoo & Pearce 2000)), reduction of the NAA peak has been used as a surrogate for axonal damage. These suggest that chronic disability itself cannot be fully explained on the basis of demyelination alone, and furthermore that there is an important correlation between chronic disability and axonal loss.

Axon loss is unlikely to have a single cause. There is strong evidence that axon loss occurs in the acute MS lesions, but it is rather less likely to have a pathophysiological impact. The reversible nature of acute relapses is more easily explained by the resolution of oedema and of inflammation, and by spontaneous remyelination (discussed later); it is less consistent with functionally significant axon fragmentation (Bjartmar & Trapp 2001;Scolding & Franklin 1998;Smith & McDonald 1999). Indeed some studies suggest 85-95% of axons need to be damaged for significant loss of function (Sabel 1997). However, axon loss in chronic MS is widespread, and one might postulate that this could impair functional reserve, and so cause symptoms at a more modest level than certain studies predict (Sabel 1997). Axon loss may start early, but it may need to accumulate for several years before making a substantial contribution to disability (Figure 1-1).

Figure 1-1 Diagram to illustrate the hypothesis of axon loss as a substrate for accumulating disability in progressive MS. Axon loss may start early and continue for years before reaching a clinical threshold, whereupon it starts contributing to disability.



The dominant cause for accumulating axonal damage is of great importance. Two major mechanisms have been proposed, and both have considerable experimental support. The first is that inflammation *per se* causes the bulk of axon damage, the second is that axon loss accumulates as a consequence of persistent demyelination. The NAA peak, a putative marker of axonal integrity, is rapidly lost in acute MS lesions, but recovery, albeit incomplete, is similarly quick. It seems unlikely that this mirrors axonal transection, though may reflect reversible axonal damage. This degree of NAA loss is not seen in central pontine myelinolysis where demyelination occurs without marked inflammation (Davie et al. 1999). Undoubtedly some axon transection and loss occurs in acute lesions alongside an inflammatory infiltrate (Bitsch et al. 2000;Kornek et al. 2000) and the inflammation almost certainly contributes to acute axonal fragmentation (Bjartmar et al. 2001;Ferguson et al. 1997;Trapp et al. 1998). However the relationship between these two has not been conclusively proven. APP, and by inference the rate of axon loss, is greater in active lesions than inactive ones (Kornek et al. 2000), which has been taken by some to support the assertion that inflammation is causative. However the complexities of the different pathological

processes in acute lesions have already been underlined and myelin loss is a major component that cannot easily be ignored.

The second possibility, that persistent demyelination causes the bulk of axon loss, might occur by several possible mechanisms. This could occur directly, through the loss of oligodendrocyte-derived trophic support (Bjartmar & Trapp 2001;Meyer-Franke et al. 1995;Scolding & Franklin 1998) or dependence on contact with their myelin sheaths (Griffiths et al. 1998), or by sustained demyelination-induced conduction block or electrical silence (Lipton 1986), or indirectly through increased vulnerability of the exposed axon to injurious agents (Raine & Cross 1989). Furthermore, the adaptations that occur to the demyelinated axolemma to restore impulse conduction may themselves predispose to later damage. Sodium channels diffuse along the internodal membrane from their aggregations at the original nodes of Ranvier, and may facilitate sodium leakage into the axoplasm. This in turn may impair outward calcium transport, leading to calcium mediated axon damage (Kapoor et al. 2003).

While both these processes probably contribute to axon damage, is it transient (albeit perhaps repeated) inflammation or chronic demyelination that is dominant, and thus should be the focus of therapeutic intervention? Axon loss does not correlate with either inflammatory cell infiltrate, tumour necrosis factor expression, nitric oxide expression, or demyelinating activity, but is related to the overall extent of myelin loss (Bitsch et al. 2000;Kornek et al. 2000). Furthermore it is seen in lesions which are demyelinated even when they exhibit sparse or no inflammation, but is rare in remyelinated lesions (Kornek et al. 2000). In that accumulating axon loss appears to be a major determinant of chronic disease, and that the course of this appears to be closely influenced neither by early inflammatory disease activity (Bitsch et al. 2000;Confavreux et al. 2000;Kornek et al. 2000) nor by even the most profound immune suppressant treatments, it seems unlikely that inflammation is wholly responsible. These arguments would favour demyelination as the dominant cause, and the key observation of axonal preservation in remyelinated lesions gives an important direction for therapeutic efforts.

The Pathophysiology of Relapse, Recovery and Progression

A characteristic feature of early MS is the remarkable spontaneous recovery in the aftermath of acutely disabling symptoms. It is worth re-emphasising that this is

unlikely to reflect axonal fragmentation, but fits much more closely with the pathophysiology of demyelination and inflammation (Smith & McDonald 1999). While certain axons are never myelinated yet are able to support slow, but secure action potentials, acutely demyelinated axons cannot. The sodium channels of denuded axons remain clustered at the nodes of Ranvier after the myelin is lost. The intervening axolemma remains bare, and without these channels, it is unable to support an action potential. Even axons with short demyelinated segments, or where the nodes have been widened by myelin damage, may fail to propagate an impulse. High cytokine concentrations also contribute to this block, either directly or through their induction of nitric oxide production. Individually these obstacles present only a relative impediment to the propagation of action potentials, but act in co-operation to block conduction (Smith & McDonald 1999).

The return of transmission could reflect the resolution of any of these factors. The characteristically rapid clinical recovery has been shown to mirror the regression of MRI determined inflammation and it is therefore likely that falling cytokine levels are important in the initial resolution of symptoms. Sodium channels also start to diffuse along the denuded axon over a few days, allowing the return of slow impulse propagation.

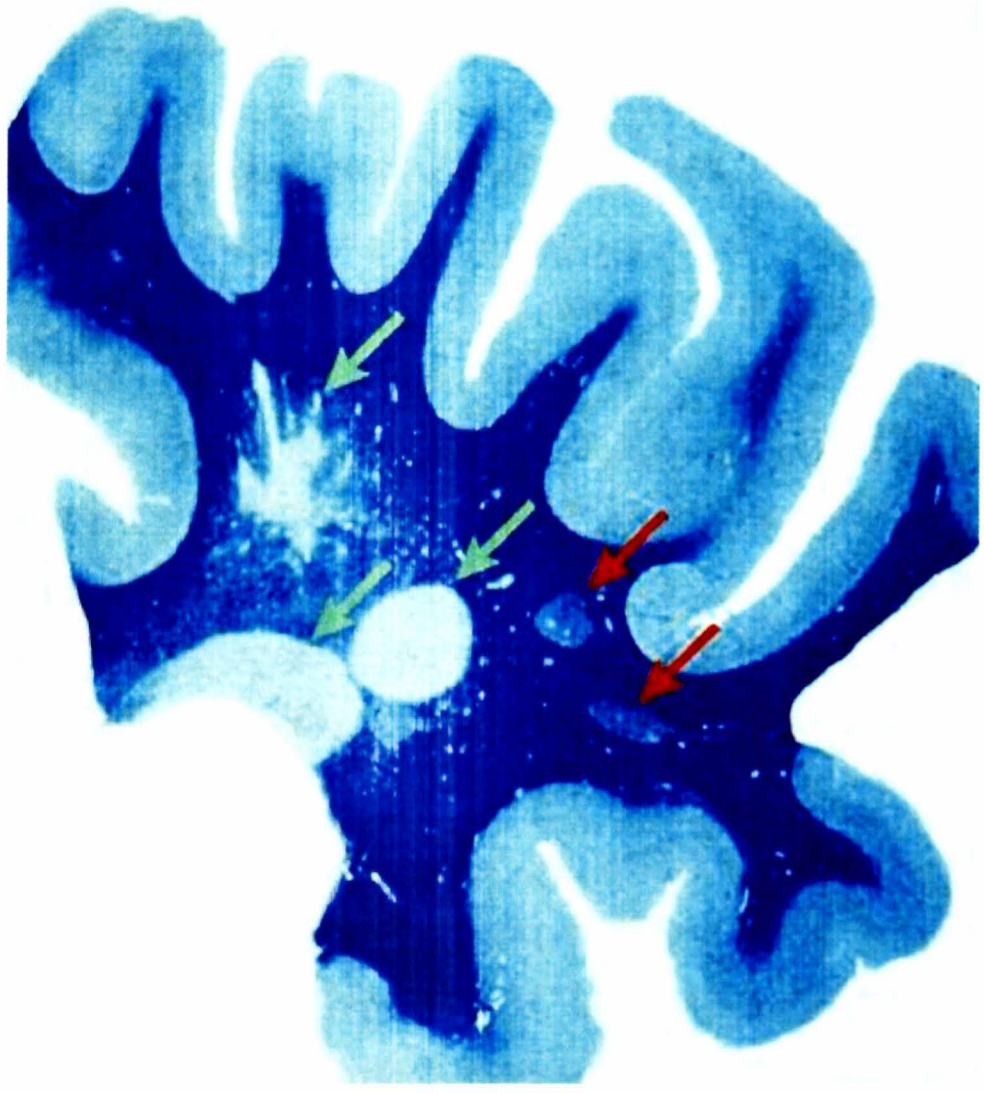
Unfortunately these reparative processes remain tenuous, and conduction block can return to persistently demyelinated axons with relatively minor physical changes or rises in systemic cytokine levels (Smith & McDonald 1999). Symptoms occurring with physical stress (Lhermitte's) or rising temperature (Uhtoff's phenomenon) both indicate that demyelination rather than axon loss is the major cause of disability in the early phase of the disease. The fact that these symptoms can also occur in chronic disease emphasises that persistent demyelination remains as important cause of symptoms even in chronic disease. It is only with repair of the myelin sheath that robust and secure saltatory conduction can return (Smith et al. 1979).

Remyelination in Multiple Sclerosis

The occurrence of spontaneous myelin repair was first demonstrated in MS in 1965 with the advent of electron microscopy (Perier & Gregoire 1965), only a few years after the first demonstration of experimental spontaneous CNS remyelination (Bunge et al. 1961). This phenomenon has been much studied by contemporary pathologists (Lassmann et al. 1997;Prineas & Connell 1979;Raine & Wu 1993) who have revealed

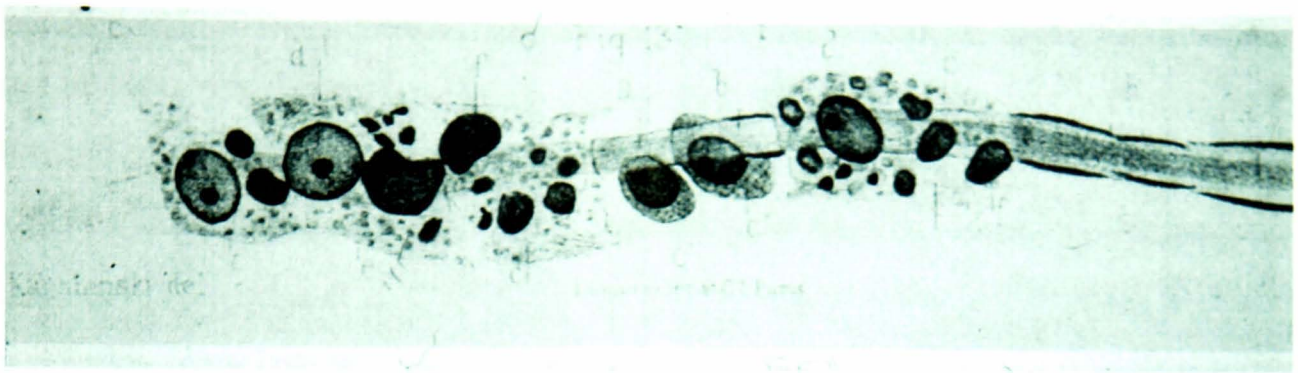
some interesting historical perspectives. Firstly, the classically described "shadow plaque" (Markschattenherde), originally felt to represent a young and active or incompletely demyelinated lesion (Dawson 1916) in fact represents successful repair of myelin across whole plaques (Figure 1-2).

Figure 1-2 Section of cerebral hemispheres of a patient who died with MS showing both persistently demyelinated plaques (green arrows) and remyelinated "shadow-plaques". Myelin labelled with Luxol fast blue. Reproduced from (Franklin 2002).



These areas are particularly evident in acute cases, are common accompaniments of immune mediated myelin damage (patterns I & II), and comprise areas of pale-staining myelin either on the edge of a plaque, or in normal unaffected white matter. They contain large numbers of uniformly thin myelin sheaths, which together with short internodes, are characteristic hallmarks of remyelination. Secondly, Joseph Babinski's often-reproduced illustration of myelin phagocytosis in multiple sclerosis unwittingly illustrates unequivocal myelin repair (Figure 1-3).

Figure 1-3 Joseph Babinski's original illustration (1885) of myelin phagocytosis in multiple sclerosis also demonstrates the characteristic thin myelin sheaths and short internodes of spontaneous myelin repair to the right of the axon.



Quiescent oligodendrocyte progenitor cells (OPC) or even more immature cells (Nait-Oumesmar et al. 1999), rather than surviving mature oligodendrocytes, are generally considered responsible for the majority of spontaneous remyelination (Gensert & Goldman 1997; Carroll & Jennings 1994;Duncan et al. 1997;Wolswijk 2000). Large populations of immature oligodendrocytes can be found in fresh MS lesions (Raine et al. 1981), including significant numbers of cells with the phenotypic markers of oligodendrocyte precursors (Chang et al. 2000a;Maeda et al. 2001;Scolding et al. 1998;Wolswijk 1998). Additionally (and particularly in the spinal cord), Schwann cells also contribute to endogenous myelin repair (Itoyama et al. 1983;Ludwin 1988;Ogata & Feigin 1975).

Recent studies have probed the origin, nature and limitations of spontaneous myelin repair. That it commences while demyelination is still occurring, as unwittingly shown by Babinski, has been confirmed (Prineas et al. 1993a;Raine & Wu 1993). In the early inflammatory phase of multiple sclerosis some 40% of plaques may contain areas of remyelination occupying 10% or more of plaque volume (Ozawa et al. 1994;Prineas et al. 1993a). This figure appears to fall as the disease progresses (Ozawa et al. 1994), with the majority of chronic plaques having either no new myelin or a thin peripheral rim (Lassmann 1983). Histological repair does not equate with functional recovery, but there is convincing evidence that central remyelination is accompanied by the restoration of secure saltatory conduction (Smith et. al. 1979). This process of repair, however effective, is limited and ultimately fails to prevent the accumulation of disability.

A number of possible explanations for the failure of repair have been proposed. Repeated demyelination appears to be associated with impaired repair in MS (Prineas et al. 1993b;Prineas et al. 1993a) and the depletion of remyelinating cells has been

proposed as a possible mechanism (Carroll et al. 1998;Duncan et al. 1997;Keirstead et al. 1998;Rosenbluth 1996;Scolding & Franklin 1999). Indeed there is much to favour this as a hypothesis although recent evidence suggests that progenitor depletion may not be a universal consequence of repeated demyelination (Penderis et al. 2003). The limited migratory capacity of endogenous progenitors may contribute (Franklin et al. 1997) and/or oligodendrocyte progenitors may be directly targeted by disease processes (Niehaus et al. 2000). However, changes in chronically demyelinated axons, rendering them less amenable to remyelinating cells, are also likely factors (Charles et al. 2000).

In addition, the progression of the astrocytic scar, obstructing the dispersal of myelin-forming glia within areas of demyelination, also might impede remyelination (Fawcett & Asher 1999). The influence of astrocytes is complex, however. In experimental studies, extensive myelination by implanted oligodendroglial lineage cells or by Schwann cells occurred unimpeded by host or purified transplanted astrocytes (Archer et al. 1997;Duncan & Hoffman 1997;Franklin et al. 1991). Others have described cohabiting astrocytes and Schwann cells in lesions which exhibit "peripheral" myelin repair (Itoyama et al. 1983;Ogata & Feigin 1975). Paradoxical roles for astrocytes are increasingly appreciated in oligodendrocyte remyelination: chronic astrocytic scars may obstruct myelin repair by oligodendrocyte progenitors, while acutely reactive astrocytes synthesise and release pro-migratory and proliferative growth factors for oligodendrocyte progenitors. A final possibility, eloquently argued in a recent review (Franklin 2002), is that dysregulation of a finely co-ordinated process, rather than failure of any one component of this, is ultimately responsible.

Whatever the mechanism of failure, persistently demyelinated axons dominate the pathology of chronic MS. If, as we have argued, chronic demyelination rather than active inflammation is mostly responsible for the progressive axonal loss of chronic disease, and axonal loss is confirmed as a major substrate of progressive disability along with demyelination-induced conduction block, then myelin repair must be a major therapeutic priority. The challenge is to augment successful, but limited, spontaneous myelin repair in time to prevent axon loss.

Strategies for repair

Myelin damage and oligodendrocyte loss, while occurring in the context of other processes, dominate the pathology, broadly explain the clinical phenomena, and may be ultimately responsible for the relentless progression of MS. They thus become enormously attractive targets for therapeutic intervention.

The aims of a remyelinating therapy are both to restore function and to protect axonal tracts from subsequent degeneration. The increasingly recognised importance of axonal loss as a pathophysiological substrate of accumulating disability by no means excludes a significant or substantial contribution by persistent demyelination *per se* to chronic disability (Scolding & Franklin 1998;Smith & McDonald 1999). Thus it should be feasible to reverse certain types of persistent disability in patients with multiple sclerosis. The relative preservation of axons even in chronic lesions offers pathological reasons for believing remyelination to have a prospect of functional value, while the transient deterioration in chronic symptoms with fever – Uhtoff's phenomenon – provides strong pathophysiological evidence for demyelination as an important substrate for impaired function.

The occurrence of spontaneous remyelination has profound conceptual implications for myelin repair therapies. The aim of such treatments ceases to be that of creating a repair phenomenon *de novo*, and becomes one of enhancing or supplementing a spontaneous process. How this might be done must self-evidently depend on understanding what may be termed the clinical biology of the disease. The reasons why endogenous repair is not even more successful, and whether the limitations can be overcome can only emerge from a fuller appreciation of the biology of myelinating glial cells.

Myelinating Glia in the Central Nervous System

Oligodendrocytes were first discovered only 80 years ago by del Rio Hortega; he also described their principal function, the synthesis of myelin in the CNS. It is now known that they develop from a progenitor cell whose properties have been much studied since culture techniques offered new opportunities for cell biological investigations (Raff et al. 1983;Skoff 1996). Furthermore, the origins of these can be traced back to cells of the developing embryo, and insights from these studies have informed our understanding of cellular specification, fate and potential. It is valuable therefore to

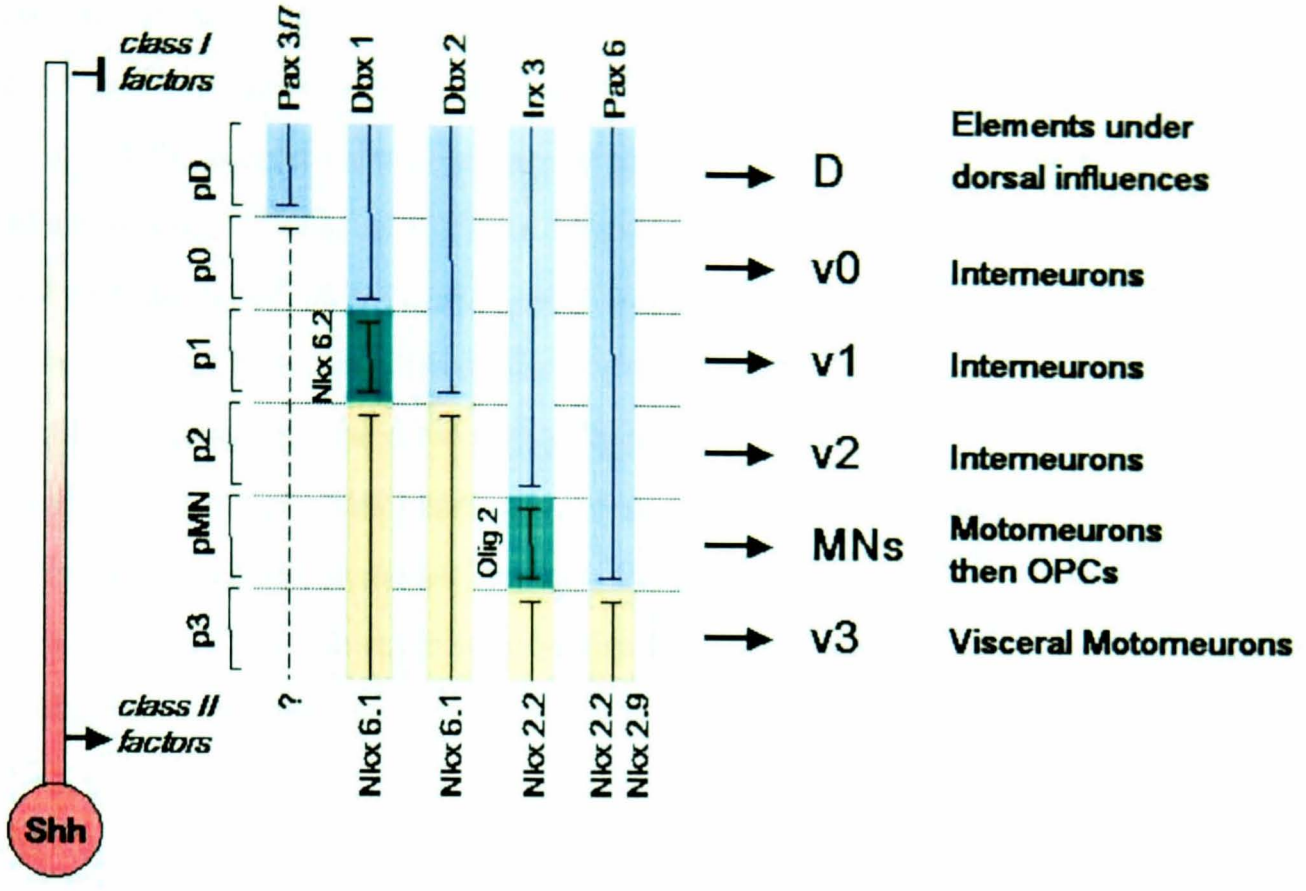
summarise some of this work as it can provide important clues as to how remyelination may be augmented.

Developmental Neurobiology *In Vivo*

Cells of the oligodendrocyte lineage are originally derived from the neural plate, an area of ectoderm that invaginates to form the neural tube in the developing embryo. Neuroectodermal cells around the primitive ventricle (ventricular zone) proliferate and migrate away from it to produce the surrounding sub-ventricular zone, developing in humans by gestational week six. Both zones contain pluripotential stem cells that divide asymmetrically to produce more lineage-restricted progenitors that populate the developing CNS parenchyma.

During development a series of waves of neuronal and glial subtypes exit the cell cycle and migrate laterally into the mantle zone where neural circuitry and glial interactions mature. The processes of cell cycle exit and lineage determination are orchestrated by the interplay of extracellular signalling molecules and nuclear transcription factors. These have been most fully understood in the spinal cord of experimental animals. A series of spatially and temporally determined gradients interact to allow the development of specific regions from which separate lineages arise. Orchestrating these processes, and of established pertinence to the production of oligodendrocytes, is sonic hedgehog (Shh), a signalling molecule secreted by cells of the notochord and overlying floor-plate. Various homeodomain transcription factors (eg Pax, Dbx & Irx proteins) are suppressed at distinct concentrations of Shh, while others are induced (e.g Nkx). Co-repression of pairs of transcription factors sharpens the boundaries, leading to a ladder-like series of zones with unique transcription factor milieus (Figure 1-4). These give rise to distinct neural progenitors destined to form different subclasses of mature neurones and glia (Marquardt & Pfaff 2001).

Figure 1-4 Transcriptional control of fate determination in the developing spinal cord. *Adapted from (Marquardt & Pfaff 2001)*



However, both motor neurones and oligodendrocytes arise from the same zone (pMN) in the ventral neural tube at different times. During motor neurone production, the bHLH factor Olig2 is induced by the co-expression of two homeodomain proteins, the Shh-activated Nkx6.1 and Shh-suppressed Pax6, where they overlap. The Olig2 expressing region is further limited by repression ventrally from Nkx2.2 and dorsally from Irx3. This combination in the Olig2 expressing region leads to the development of motor neurones, in part through Neurogenin 2 (Ngn2) a pan-neuronal determinant. Lateral inhibition of Ngn through Notch-Delta signalling leaves undifferentiated progenitors remaining in this region for subsequent glial production.

Later in development, and perhaps as a result of increased Shh secretion, the boundary between the Olig2 and Nkx2.2 expressing regions breaks down, with the latter region moving dorsally to overlap with Olig2. The expression of both Olig2 and Nkx2.2 seem to be required for myelin gene expression and oligodendrocyte production (Fu et al. 2002), but not necessarily fate determination, since some oligodendrocyte progenitors arise and migrate out from both the ventral (P3) and dorsal (pMN) regions prior to dual expression. Most research has focussed on rodent and chick models – which vary

in the precise sequence of these events, and to date these have not been clarified in humans.

Generation of oligodendrocytes in the central nervous system is less comprehensively understood but there appear to be a number of similarities to the process described above. Oligodendrogenesis appears in focal regions of the ventral forebrain, and at least some of the oligodendrocytes appear to develop form migratory cells in a Shh-dependant process (Richardson 2001). Some of this knowledge arises from the regional expression of early myelin genes and putative oligodendroglial markers. However different markers label different populations, raising the possibility of different lineages within the developing forebrain.

Oligodendrocyte progenitors generated by mechanisms such as these migrate into the developing grey and white matter regions. Unlike the essentially post-mitotic neurones, these oligodendrocyte progenitors remain mitotically active and undergo substantial further proliferation in the developing parenchyma (Marquardt & Pfaff 2001).

Proliferation and Population Control of Oligodendrocytes

As has been noted, oligodendrocyte progenitors migrate out of the subventricular zone as mitotically competent cells. These cells continue to divide in the developing grey and white matter tracts under the influence of both soluble growth factors and extracellular matrix ligands. PDGF-AA, acting through the PDGFα receptor plays an important role in both proliferation and survival of oligodendrocyte progenitors (Noble et al. 1988). Secreted by neurons and astrocytes, this molecule appears to be present in limiting concentrations (Calver et al. 1998): PDGF-A knockout mice have profoundly reduced numbers of progenitors (Fruttiger et al. 1999) and augmented production during development causes a substantial increase in progenitor numbers (Fruttiger et al. 2000). Excess numbers then die through apoptosis (Calver et al. 1998).

Recent insights into the mechanism of action of PDGFAA, obtained through *in vitro* work, suggest that at physiological concentrations, its response depends on downstream signalling between cell surface integrins and specific extracellular matrix ligands. When PDGF binds to its receptor on the surface of oligodendrocyte progenitors, in-out signalling mechanisms prime $\alpha_v\beta_1$ integrins to recognise vitronectin. Only in the presence of appropriate binding will subsequent intracellular signalling cascades lead to cellular proliferation. However as progenitors mature,

developmental switching of the β-subunit occurs leading to the expression of $\alpha_v\beta_5$, which has both a different extracellular ligand (merosin, a laminin subtype expressed in axonal tracts) and a different associated intracellular signalling cascade. The same signal that promotes cell division and inhibits differentiation then promotes survival and maturation (Baron et al. 2002).

Following the proliferative phase, the majority of oligodendrocyte progenitors that fail to associate with axons die by apoptosis. This is particularly evident in models where over-expression of PDGF has lead to a significant excess of these cells (Fruttiger et al. 2000). Widespread cell death in these animals leads to a similar numbers of mature oligodendrocytes as is seen in wild-type controls. Integrin signalling may go some way to providing mechanisms for this type of population control (*vide supra*). However, oligodendrocyte progenitors persist in the adult rodent, and undergo slow turnover (Alonso 2000;Horner et al. 2000). Why these cells remain in the undifferentiated state in the adult nervous system is unclear. Their prime role may be oligodendrocyte replacement and to facilitate remyelination following injury but there is growing evidence of additional physiological roles for oligodendrocyte progenitors. Oligodendrocyte progenitors in the rodent hippocampus receive substantial numbers of synapses directly from neurons (Bergles et al. 2000), and may themselves modulate inter-neural signalling (Levine et al. 2001). The reasons for these interactions remain broadly speculative.

A possible mechanism for how these cells maintain this quiescent state arises from the observation that mature central nervous system myelin arrests maturation of oligodendrocyte progenitors (Miller 1999). It seems possible that these cells, following migration into the CNS parenchyma, arrive after the formation of significant quantities of myelin, and so are held in the undifferentiated state. The influence of the integrin-mediated interactions with axons and extracellular matrix on cytokine response, discussed earlier, may also play a role (Baron et al. 2002).

The Oligodendrocyte Lineage

The exact genealogy of glia, often studied using cell culture and transplantation, has been much harder to determine. There may be fundamental reasons for this difficulty. Traditional dogma suggests that cells progress in an orderly sequence from the totipotential cells of the early blastomere, through progressively restricted stem cells of the ventricular and sub-ventricular zone and then via transient amplifying cells to

the final maturation pathway of the post-mitotic oligodendrocyte. Numerous antigenically distinct glial progenitors have been described *in vivo* and *in vitro*, and the apparent differentiation potential of many of them has been well documented (for review see (Lee et al. 2000). However, a number of recent findings have challenged the traditional model. The unexpected potential of apparently tissue-restricted stem cells (Alison et al. 2000;Bjornson et al. 1999;Clarke et al. 2000b) and reports suggesting de-differentiation of lineage-restricted progenitor cells (Kondo & Raff 2000a) have suggested that, at the very least, there may be exceptions to this "rule". The significance not only of a cell's history, but also of its niche, influential not only to its differentiation pathway but also to the maintenance of a stem cell's phenotype, is becoming appreciated (Temple 2001). Furthermore the importance (and difficulty) of distinguishing between a cell's fate *in vivo* and potential *in vitro* is increasingly apparent; the degree to which cellular plasticity is mirrored in physiological or pathological conditions *in vivo* is unclear.

In spite of these important caveats, and the large number of apparently different classes of glial progenitors described, a broadly coherent path of rodent glial differentiation can be traced from neural stem cell through to mature myelinating oligodendrocyte. Significantly less information is available from human work, but some investigators, appreciating the hazards of ignoring the species barrier, have attempted to redress this (Ostenfeld et al. 2002).

Stem Cells

Before discussing the glial lineage, it is worthwhile clarifying some general points about stem cells. Stem cells are defined as clone forming, self-renewing progenitor cells that can generate one or more specialised cell types. There are several different types that can be divided into those that are active during development and those that have a primarily regenerative role. These cells often express DNA repair enzymes such as telomerase, which help to defer cellular senescence and possess unusual growth characteristics, including the ability to divide asymmetrically, although symmetric division can also be seen during development (Temple 2001).

Embryonic Stem Cells

This group includes the only truly totipotent cells (able to give rise to all cell types including those of the trophoblast) which are the fertilised egg and the cells of the early developmental blastomere, and they are merely mentioned for completeness. Pluripotent cells (able to give rise to all cell types of the developing embryo and adult), which are found in the inner cell mass of the developing embryo, were first isolated in 1981 (Evans & Kaufman 1981). This followed on from work on teratocarcinomas, tumours which contain elements derived from all three embryonic layers; ectoderm, mesoderm and endoderm. These cells are highly proliferative and pluripotent, but carry with them the risk of teratoma formation following transplantation. Much of the initial work on these cells was performed in rodents, but they have subsequently been isolated from human embryos, grown in culture (Thomson et al. 1998) and can be enriched for neuronal lineages using a combination of growth factors and mitogens (Carpenter et al. 2001). Similar cells migrate widely when placed in the developing rat brain, and differentiate appropriately without the development of teratomas (Zhang et al. 2001). Other investigators have demonstrated that embryonic germ cell lines obtained from aborted human fetuses carry a similar potential to pluripotential embronic stem cells (Shamblott et al. 1998).

Neural Stem Cells

As development progresses, embryonic stem cells give rise to a second group of cells which retain the essential characteristics of stem cells, but whose progeny develop into cells of one particular organ or tissue (organ-specific stem cells). These cells are still multipotent (able to give rise to more than one cell type) and highly proliferative, and can be isolated from many different tissues.

Cells have been isolated from embryonic rodent brain, expanded with epidermal growth factor, subjected to clonal and population analysis and shown to possess the key attributes of a stem cells (Reynolds & Weiss 1996). Interestingly, subsequent work suggests that more primitive rodent neuroepithelial stem cells initially do not express EGF receptors (Kalyani et al. 1999) but may develop them in response to FGF stimulation (Temple 2001). There may also be differences in the default pathway of differentiation at different stages of development; embryonic stem cells favour neuronal progeny, while later in development astrocytes appear to be the default

pathway (Zhu et al. 1999). This exemplifies the difficulties inherent in interpreting studies where different sub-populations may exist in close proximity.

Embryonic neural stem cells have unusual growth characteristics in that they start to proliferate in an anchorage-independent fashion and continue to proliferate as colonies. Cultures of these cells, with appropriate mitogens develop free-floating aggregates termed "neurospheres". This growth pattern is mirrored by other stem cells and has facilitated their isolation and identification, particularly in that most other neural cell types grow only as adherent cultures. Aggregate culture systems are believed to exploit cell-cell, and possibly cell-matrix interactions, which are as yet imperfectly understood. Daughter cells derived from these spheres have a tendency to start differentiation after plating down (Reynolds & Weiss 1996). Important mitogens for these cells include FGF2 & EGF (Reynolds & Weiss 1996), either separately or in combination, although autologous secretion of FGF complicates attempts to dissect the exact growth requirements of these cells. They characteristically express nestin, an intermediate filament protein originally identified as a marker of neuroepithelial precursors (Frederiksen & McKay 1988).

These cells have also been identified in the developing human brain and proliferate in response to EGF & FGF2 alone (Vescovi et al. 1999), or in combination with leukaemia inhibitory factor (LIF) (Carpenter et al. 1999;Uchida et al. 2000). More recently these cells have been demonstrated from all regions of the developing rodent and human brain, but they do not appear to be entirely homogeneous. Furthermore, important interspecies differences have again been demonstrated (Ostenfeld et al. 2002).

Adult Stem Cells

The presence of stem cells in adult mammals was surmised in organs such as skin and blood where cell turnover continues at a high rate throughout adult life. They were first identified, and perhaps most comprehensively characterised in the haemopoetic system, and this knowledge continues to inform our understanding of stem cell biology. Adult stem cells play a very different role from their developmental counterparts, but the characteristics of self-renewal, clone formation and multiple lineage potential remain. They act as a source of cells lost during normal cell turnover, as epitomised by the haemopoetic system, but they are also able to orchestrate regenerative repair following trauma or disease. Perhaps the most dramatic example of

this is seen in certain amphibians that retain the ability to regrow entire limbs. This ability is attributed to small numbers of cells that proliferate and differentiate rapidly from the stump following trauma. The best examples of regenerative repair in humans occur in the skin and liver, although even in these organs the cues that orchestrate repair are poorly understood.

Adult stem cells are a numerically small group of cells and they possess unusual qualities that set them aside from other cells of the mature organ. The ability to divide asymmetrically; that is to produce an identical copy of themselves as well as a more committed daughter cell, is one of these. Furthermore, they possess a phenomenal replicative potential that enables them to repopulate large areas of damage, and to do this may activate DNA repair enzymes, such as telomerase, which defer senescence. Adult stem cells appear to be relatively robust, a necessity in a cell whose vocation it is to survive disaster and repopulate. This is exemplified by their capacity to survive both enzymatic dissociation protocols and protracted post-mortem intervals (Laywell et al. 1999). This may be a consequence of relative metabolic inactivity as they often reside in a quiescent state, yet they are able to respond rapidly and dramatically to the challenge of repopulating damaged tissue.

These cells have been identified in diverse organs such as bone marrow, skin, liver and muscle where regeneration is prominent. However regenerative repair is rare in the central nervous system, remyelination being a crucial exception to this, so their isolation from the adult rodent brain was of particular note (Gritti et al. 1996;Rietze et al. 2001;Weiss et al. 1996). In fact neurogenesis also continues throughout adulthood, with cells destined for the hippocampus and rostral migratory stream. However demonstration of these adult neural stem cells has fuelled increased interest in the possibility of more widespread CNS regeneration in response to injury or disease.

Adult Human Stem Cells

More recently the isolation of stem cells and their growth *in vitro* from adult human brain has also been reported (Arsenijevic et al. 2001;Johansson et al. 1999;Kukekov et al. 1999;Palm et al. 2000) although rigorous demonstration of clonogenicity and of functional progeny from separate neural lineages, or prolonged expansion *in vitro* is not always presented.

These cells have similar characteristics to their fetal counterparts, expressing nestin and Musashi-1 (Palm et al. 2000). They grow into spherical bodies in free-floating

cultures, under the influence of EGF and FGF2, and differentiate into cells bearing markers of astrocytes, neurons and oligodendrocytes. Interestingly, though multipotent, oligodendroglia are poorly represented among progeny – typically 1-5% of all daughter cells express GalC (Palm et al. 2000). However there are some notable differences between adult and fetal human stem cells, notably the former's ability to differentiate even in the presence of mitogens (Arsenijevic et al. 2001). If confirmed, the isolation of human stem cells form adults has far-reaching implications.

The Generalised Potential of "Organ-Restricted" Stem Cells

As our knowledge has extended, traditional assumptions as to organ restriction have been challenged. The first reports of transdifferentiation emerged with the discovery of donor-derived myocytes occurring in the recipient following bone marrow transplants (Ferrari et al. 1998). This observation lead to a several experiments into the lineage potential of diverse stem cells. These have uncovered many such example of transdifferentiation (Alison et al. 2000;Azizi et al. 1998;Bjornson et al. 1999;Galli et al. 2000), including the extraordinary generalised potential of adult neural stem cells when implanted into blastocysts, where they can generate cells from all three germ layers (Clarke et al. 2000a). Of particular note is the discovery of the neural potential of bone marrow derived cells (Mezey et al. 2000a), mesenchymal stem cells (Woodbury et al. 2000) and possibly their haemopoetic counterparts (Bonilla et al. 2002), discussed later.

Glial-Restricted Progenitors and Oligodendrocyte Pre-Progenitors in Rodents

From the early neural stem cell, it is likely that progeny go through a stage of restriction to a glial fate before further commitment to oligodendrocyte differentiation, although this hypothesis remains unconfirmed in humans. Cells able to produce both astrocytes and oligodendrocytes, but without evidence of neuronal differentiation have been described from rodent embryonic spinal cord (Rao et al. 1998;Rao & Mayer-Proschel 1997). These cells express nestin (Gallo & Armstrong 1995)and appear to gain PDGFαR expression as they mature (Lee et al. 2000). Other researchers, also attempting to clarify whether oligodendrocytes and astrocytes emerge from a common progenitor, have described a cell population with a slightly different antigenic phenotype and response to growth factors that again seems restricted to glial progeny (Ben Hur et al. 1998).

Pre-progenitors, originally defined as the immediate precursors of the "O-2A" stage (*vide infra*) express PSA-NCAM, nestin and both PDGFαR and FGF receptors. These cells appear to be able to form spheres with FGF2, while both FGF2 and tri-iodothyronine are important survival factors. Withdrawal of these mitogens leads to the production of astrocytes and oligodendrocytes (Ben Hur et al. 1998). To what extent these cells could represent stem cells that have not manifested their neural potential because of culture conditions is unclear, but stem cells much more readily produce neurons than oligodendrocytes (Palm et al. 2000).

A direct human equivalent to this cell has yet to be identified. However, studies of human oligodendroglial production from fetus-derived sphere forming cells have been performed and followed through to myelin basic protein-expressing oligodendrocytes (Zhang et al. 2000).

Rodent Oligodendrocyte Progenitors – the "O-2A" Stage

One of the most studied cells of the oligodendrocyte lineage is the rodent "O-2A" progenitor – so called because of its potential for differentiation into oligodendrocytes or a type of astrocyte (designated "type 2") *in vitro*. It was first identified in tissue culture from neonatal rodent optic nerve (Raff et al. 1983) and subsequently from other parts of the postnatal CNS. These cells differentiate constitutively into oligodendrocytes on mitogen withdrawal, but can be induced to produce "type 2" astrocytes by certain environmental cues. "Type 2" astrocytes express certain ganglioside antigens (including A2B5 epitopes) not expressed by astrocytes *in vivo* and lack Ran2 epitopes (Lee et al. 2000). They seem not to be formed in normal maturation, and transplantation of the O-2A cells into developing rodents sees the cells differentiate exclusively into oligodendrocytes, and participate in myelination (Groves et al. 1993). However, there is some evidence that the type 2 astrocyte pathway can be induced *in vivo* in certain pathological situations (Matthews et al. 1995).

O-2A cells are highly motile with two long processes when seen under phase contrast. They carry a characteristic immuno-phenotype, with surface molecules (subsequently shown to be gangliosides) recognised by the A2B5 and anti-GD_3 antibodies. They have also be identified by the presence of the PDGFα receptor (Pringle et al. 1992;Yu et al. 1994), or the related surface component chondroitin sulphate proteoglycan (Stallcup & Beasley 1987), or by their characteristic glutamate channel properties (Fulton et al. 1992) (discussed further in Chapter 4). O-2A cells have not been thought

to generate neurons or type 1 astrocytes except under exceptional circumstances (Kondo & Raff 2000b).

Rodent neonatal "O-2A" progentiors are influenced by a number of different mitogens. Fibroblast growth factor 2 (FGF2) (also known as basic FGF) is a member of a structurally related family of 14 polypeptides. Present in developing and adult brain it has a broad range of mesodermal and neuroectodermal cell targets. Four high affinity FGF receptors and some variants have so far been identified along with a class of low affinity, high capacity cell surface receptors, heparan sulfate proteoglycans, which are essential for signalling. A number of cell adhesion molecules can also interact with interact with these receptors (eg L1, NCAM, N-Cadherin) (Bansal & Pfeiffer 1997).

FGF2 plays a prominent role in oligodendrocyte development. It up-regulates PDGFα-receptor expression on early progenitors, and in combination with $PDGF_{AA}$ supports long term proliferation and migration (Bogler et al. 1990;McKinnon et al. 1990;Milner et al. 1997;Osterhout et al. 1997). FGF2 is mitogenic for late progenitors and reversibly blocks their terminal differentiation (Bansal & Pfeiffer 1994;Gard & Pfeiffer 1993;McKinnon et al. 1990). During lineage progression, oligodendrocytes regulate the expression of their FGF receptors. Progenitors express FGF-receptors 1,2&3, and upregulate FGF-R3, heparan sulfate proteoglycan coreceptors(Syndecan1,2,3&4) and glypican during terminal differentiation (Bansal & Pfeiffer 1997).

The second major mitogen acting on rodent progenitors is platelet derived growth factor $PDGF_{AA}$. This polypeptide induces proliferation of glial precursors (Calver et al. 1998;Engel & Wolswijk 1996) acting on the PDGFαreceptor, which is expressed at pre-progenitor stage (*vide supra*). It maintains oligodendrocyte progenitor cells in a proliferative phenotype and promotes cell division and motility (Engel & Wolswijk 1996).

Several other mitogens, hormones and second messengers have a role in glial development. Neurotrophin-3 (NT-3) promotes neonatal rodent oligodendrocyte progenitor survival, and synergises with the mitogenic effect of $PDGF_{AA}$. Leukaemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) induces O2A cells to differentiate into oligodendrocytes and then acts as a survival factor (Mayer et al. 1994).

Oligodendrocyte progenitors express the chemokine receptor CXCR2 and its ligand, Groα enhances O2A proliferation in the presence of $PDGF_{AA}$ (Nguyen & Stangel 2001). Neuregulins may be required for proliferation and survival of later oligodendrocyte progenitors and pro-oligodendrocytes (Canoll et al. 1996). Tri-iodothyronine and retinoic acid promote oligodendrocyte differentiation and inhibit proliferation (Barres et al. 1994;Billon et al. 2001), and increased intracellular cyclicAMP accelerates oligodendrocyte precursor differentiation (McKinnon et al. 1990;Raible & McMorris 1989).

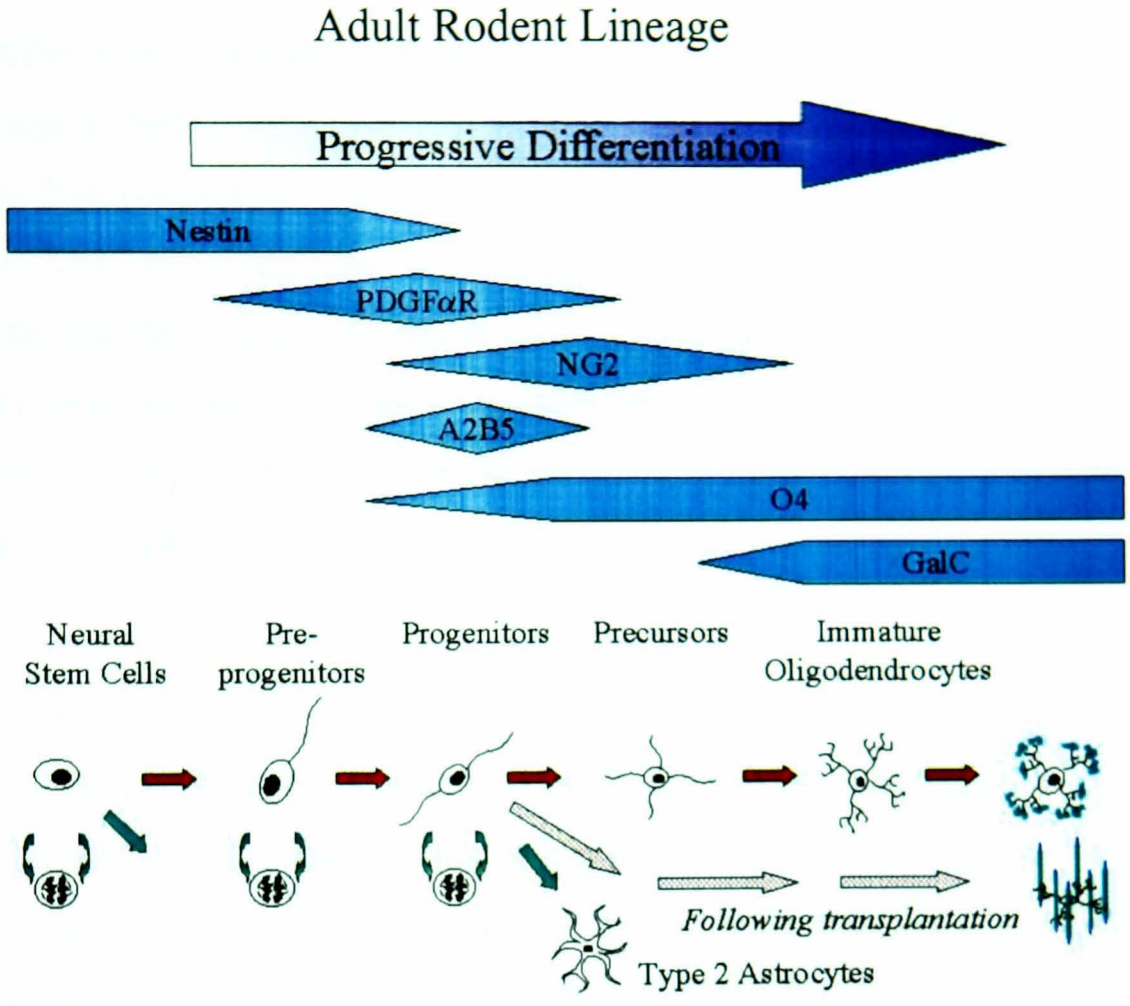
Finally, an intriguing hypothesis based on a final common pathway of intracellular redox state has been proposed in rodent progenitors; the more reduced a cell, the more likely it is to proliferate. Thus mitogens which promote proliferation induce a reduced intracellular environment while those promoting differentiation promote a more oxidised state (Smith et al. 2000).

The growth characteristics of perinatal rodent O-2As are complex. Mathematical analysis suggests that they undergo both symmetric and asymmetric division at different times, perhaps requiring a critical period of symmetric division to attain asymmetrical division-competence (Whittemore et al. 1994). Thyroid hormone and the bHLH gene Id4 appear to play important roles in this regard, both in the clock mechanism and the subsequent differentiation into oligodendrocytes (Kondo & Raff 2000). As this process continues the development of an adult type progenitor emerges [*vide infra*] with distinct biological properties.

Fetal Human Oligodendrocyte Progenitors

Equivalents to this cell have been isolated from fetal human brain (Kennedy & Fok-Seang 1986) but interestingly attempts to grow these or related cells with the rodent mitogens PDGF and/or FGF2 have shown little response (Aloisi et al. 1992). Sphere-derived human embryonic progenitors differentiate more slowly than their rodent counterparts and divide more rapidly in contact with astrocytes or neurons, but follow the same pathway (Zhang et al. 2000).

Figure 1-5 A Comparison between the adult rodent and human oligodendrocyte lineage reveals broad similarities, but important interspecies differences. Much of the rodent work has yet to be confirmed with human cells



Adult Rodent Oligodendrocyte Progenitors

A cell with similar potential also persists in the adult rodent nervous system, although this cell is intrinsically different from its perinatal equivalent, with subtle but important differences in antigenic phenotype and mitogenic response (Shi et al. 1998;Wolswijk & Noble 1989). The neonatal cells can be discriminated by the intermediate filament protein vimentin, which is absent from the adult form (Wolswijk et al. 1991b). The latter also divide much more slowly, are less migratory and usually unipolar, although they can be induced to revert to neonatal phenotype (Wolswijk & Noble 1992). Both the neonatal and the adult forms of the rodent cell have mitotic potential, proliferating markedly under the influence of PDGF and FGF2 as well as other mitogens (Bogler et al. 1990).

Adult Human Oligodendrocyte Progenitors

Following a substantial number of studies identifying adult oligodendrocyte progenitors in rodents, the existence of an equivalent cell in the adult human was first suggested by the isolation of a bipotential oligodendrocyte progenitor from adult human white matter (Scolding et al. 1995) and confirmed later by tissue print experiments (Scolding et al. 1999). These studies indicate that this oligodendrocyte progenitor is a small bipolar cell and labels with A2B5. Furthermore, it develops into an oligodendrocyte in low serum conditions, and into a "type 2" astrocyte at higher serum concentrations. This cell does proliferate modestly on an astrocyte monolayer; cells incorporating bromodeoxy-uridine (BrDU) and hence passing through S-phase were identified, but no proliferation was seen with PDGF, FGF or NT-3 alone or in combination, mitogens known to stimulate adult rodent progenitor proliferation. Since then cells with similar characteristics have been further studied by selecting out fluorescent cells from adult human primary glial cultures transfected with a gene encoding for green fluorescence protein, driven by an early promoter for an oligodendrocyte specific protein. These cells also stained with A2B5, but no evidence for subsequent proliferation *in vitro* was presented (Roy et al. 1999).

This stage of the oligodendrocyte lineage is of particular interest. As discussed earlier, intrinsic remyelination is widely accepted as being mediated by oligodendrocyte progenitors at the "O-2A" stage (referred to simply as "oligodendrocyte progenitors" in the following chapters) that proliferate and migrate into lesions. The process of

myelin formation has to start while the cell is still dividing (Compston et al. 1997;Keirstead & Blakemore 1997) and subsequent stages in humans appear not to divide (*vide infra*). Furthermore, the most efficient myelinating population in rodents appears to be comprised of O-2A progenitors (Keirstead et al. 1999;Warrington et al. 1993;Zhang et al. 1998a), with little or no myelination occurring with transplantation of human GalC positive immature oligodendrocytes or later (Targett et al. 1996). More primitive cells may need to be committed to the oligodendrocyte lineage prior to transplantation (Smith & Blakemore 2000).

In stark contrast to the wealth of investigations that have used rodent cells, very few have studied adult human oligodendrocyte progenitors, and those that have demonstrate differences that highlight the species barrier (Armstrong et al. 1992;Prabhakar et al. 1995;Scolding et al. 1995). Further information about these important human cells is urgently required.

Rodent and Human Oligodendrocyte Differentiation and Maturation.

The next identifiable stage of the differentiation pathway in rodents is termed the pro-oligodendrocyte. These cells develop a more complex morphology with multiple branched processes and start expressing epitopes, such as those on sulfatide, recognised by the O4 antigen (Gard & Pfeiffer 1993). This cell remains modestly proliferative, although its migratory potential is more limited, presumably reflected in its altered morphology.

The identification of antigenically similar cells in adult human brain was an early indicator that oligodendrocytes might arise in the mature brain parenchyma (Armstrong et al. 1992) and that sources of myelin other than mature oligodendrocytes might be responsible for intrinsic remyelination in humans. However this cells does not proliferate in culture, and was termed the pre-oligodendrocyte to differentiate it from its proliferative rodent counterpart.

As cells mature beyond this point they become essentially post-mitotic and start expressing galactocerebroside (GalC). Both O4 and GalC are considered reliable markers of oligodendrocyte lineage cells both *in vivo* and *in vitro* (Ranscht et al. 1982), although occasional cells with both GFAP and GalC staining have been reported (Raff et al. 1983;Scolding et al. 1999).

Post-mitotic rodent oligodendrocytes cells then follow an orderly sequence of myelin gene expression to the fully differentiated oligodendrocyte (Compston et al. 1997), a

sequence that appears to be broadly similar in humans (Zhang et al. 2000). If exposed to appropriate conditions early enough in this development the cells will make contact, engage and produce myelin membranes which ensheath and finally compact to form mature functional myelin segments. This is a complex interaction requiring the presence of a combination of physical factors, including the presence of appropriately sized axons, cell-cell and cell-matrix interactions and soluble ligands (Compston et al. 1997;Zajicek & Compston 1994).

Investigative Strategies

Remyelinating therapies have enormous potential in the treatment of MS, but rely on a detailed understanding of the clinical biology of the disease in general, and the human oligodendrocyte progenitor in particular. This is broadly lacking. An enormous amount is known about the rodent oligodendrocyte lineage but significant differences exist between these species. We have argued strongly that information from work with rodent cells cannot be reliably extrapolated to humans without direct experimental support using human cells. Such studies require a large number of cells. Furthermore, there are good reasons for believing that the adult human oligodendrocyte progenitor is the best candidate for any transplantation strategy in MS. If this potential is to be realised, then even larger numbers of cells will be required.

The predominant obstacle to the investigation of the human oligodendrocyte lineage is the limited number of cells available for experimentation. Various sources of human glia exist – and some of them have been used experimentally. Many experiments have used tissue derived from aborted fetuses, an approach that carries significant ethical problems. Tissue from spontaneous abortions, while more ethically acceptable, involves its own complex issues of consent.

The major sources of adult cells are surgical specimens from patients undergoing treatment for refractory epilepsy. These samples typically contain less than $1cm^3$ in volume (own observations) from which of the order of 2000 oligodendrocyte progenitors can be harvested (Roy et al. 1999). Other tissue such as that resected during emergency frontal and temporal lobectomies for life-threatening cerebral oedema has also been used, but most investigators have been careful to avoid specimens from tumour surgery because of the risks of confusing resident glia with neoplastic cells. The use of adult post-mortem tissue, while providing sizeable starting volumes, is limited by marked reductions in cell viability, although there are

suggestions that some of the more immature precursors may survive for long periods post-mortem (Laywell et al. 1999;Palmer et al. 2001;Roisen et al. 2001).

The absence of techniques to expand the limited starting numbers confounds this problem. Occasional human oligodendrocyte progenitors cells divide on astrocyte monolayers, but these cells have not been expanded significantly *in vitro*. Thus one of the foremost issues for which human tissue is so necessary, understanding their mitogen requirements, provides the very obstacle to its investigation.

It is this particular problem that the following series of experiments addresses. Four approaches have been explored to overcome this hurdle. Chapter 2 describes the investigation of primary human glial progenitors previously transformed using viral oncogenes to enable controlled proliferation. Chapter 3 describes the strategy of dedifferentiating "committed" progenitors to a more proliferative state. Chapter 4 describes the study of primary adult human oligodendrocyte progenitors and their expansion in cell culture using mitogens and Chapter 5 describes the isolation and expansion of adult neural stem cells and strategies to induce differentiation along the oligodendrocyte lineage.

Chapter 6 addresses a specific question of relevance to our understanding of the clinical biology of MS, and the role of inflammation in remyelination. It also illustrates the need for substantial numbers of cells, and exemplifies the type of study required to understand this debilitating disease.

Hypothesis

The starting hypothesis for these experiments can be summarised as follows: Human oligodendrocyte progenitors and neural stem cells are present in, and can be isolated from adult human brain. They can be grown *in vitro* and can be expanded, either using genetic manipulation or with mitogens predicted from studies using other mammalian glia. They can provide a source of substantial numbers of oligodendrocyte progenitors for studying oligodendrocyte biology. In addition they may represent a practical source of cells for transplantation studies, and ultimately could provide a source of cells for therapeutic transplantation.

Aim

The goal of these experiments was to help develop remyelination therapies by providing insights into the biology of the adult human oligodendrocyte progenitor. The

major hurdle was thus to find a source of substantial numbers of purified human oligodendrocyte progenitors with which to answer the many questions of oligodendrocyte biology.

Chapter 2. An Investigation of the Human Oligodendrocyte Progenitor Cell Line HW1

Introduction

Directed transformation of a human oligodendrocyte progenitor to form an immortalised cell line is an attractive solution to the problem of cell numbers. This approach has been used successfully with other myelinating glia (Barnett et al. 1993). The use of conditional oncogenes such as the temperature sensitive mutant of SV40 large T antigen incurs added benefits. With the oncogene active, the normal controls to cell proliferation are bypassed, allowing large numbers of cells to be grown with relative ease. By switching off the oncogene, the cell can revert to its original phenotype, rewarding the researcher with vast numbers of genetically and phenotypically identical cells.

Producing cell lines using SV40 antigens is a valuable and frequently used procedure and the mechanism with which these cells overcome blocks in the cell cycle have been a subject of much scrutiny (Bryan & Reddel 1994;Jha et al. 1998). These mechanisms are not uniform, varying significantly between lines derived from different cell types and possibly even between lines from the same source cell. It appears likely that in most cells, immortalisation is a multi-stage phenomenon, some stages of which may be stochastic or only indirectly related to the action of the oncogene product. The most common pattern seen with large T antigen is for the gene product to cause a limited delay of cell senescence, after which the population passes through a "crisis". During this, the majority of cells apoptose, while a much smaller sub-population survives to become truly immortal. Presumably this involves a further genetic event, such as the loss of a further tumour suppresser gene. However, the proportion of cells surviving crisis varies considerably, and some cell lines (Bartek et al. 1991), including those created with the temperature sensitive double mutant used here(Barnett, S. personal communication) show no evidence of passing through crisis.

A previous investigator, Dr H Wilson, created the original human oligodendrocyte progenitor cell line used in this investigation. This cell line, which will be referred to as HW1, was created by infecting a primary human glial culture with a retroviral vector carrying the temperature-sensitive double mutant of SV40 large T antigen. The resulting cell was characterised and reported at a recent conference.

In brief, it was shown to possess an immunophenotype comparable to that of early oligodendrocyte lineage cells. When grown at 33°C (the permissive temperature), the cells stained positively with A2B5 and O4 antibodies, and with antibodies directed against NG2 chondroitin sulphate. Immunofluorescence using antibodies directed against astrocytes (GFAP), more mature oligodendroglia (GalC, MOG, MBP), more immature stem/progenitor cells (nestin, vimentin) and neurons (β_{III} tubulin, MAP2 & neurofilament) were negative, as were antibodies identifying other cell types including fibroblasts (Thy1), microglia (CD11b), Schwann cells (S_{100}, NGF receptor) and endothelial cells (von Willebrand factor).

Surprisingly, immunocytochemistry for PDGFα receptor was negative. However, SDS-PAGE and Western blotting, using monoclonal anti-PDGF receptor antibody as the probe, demonstrated that it was present in low quantities.

When cultured at a temperature sufficient to inactivate the oncogene (39°C), cell division was reduced and some cells demonstrated morphological evidence of differentiation and expressed the later lineage marker GalC (Figure 2-1).

This cell line was frozen in a standard manner in Cambridge, stored in liquid nitrogen, and then transferred to the cryostorage facility in the Glial Cell Biology Laboratory (either directly, or via the cryostore at the pathology department).

Plan of Investigation of HW1

Further Characterisation of the Cell Line

The growth properties and antigenic profile of HW1 needed repeating to confirm its integrity as an oligodendrocyte progenitor. Its stability, and the cessation of oncogene activity, also needed further clarification, as preliminary evidence indicated that only a limited proportion of cells left the cell cycle and commenced terminal differentiation at the non-permissive temperature (H.Wilson, personal communication).

Directed Experiments Using the Cell in its Untransformed State

Experiments were planned to investigate the cell following inactivation of the oncogene by growth at 39°. These aimed to investigate stimuli and inhibitors of cell division and differentiation, migratory potential at different stages of differentiation and on different substrates, chemokinesis, and finally myelin formation. Two approaches were anticipated, the former approach would test the hypothesis that

rodent and human cells behave similarly. The second would be to probe for growth factor receptor expression using immunocytochemistry and molecular biological techniques, and then to use this data to identify potential mitogens.

Co-culture Experiments

The simultaneous growth of HW1 and neuronal cell lines would be used to determine the biological interactions involved in myelination.

Transplantation Studies

It would be anticipated that success in the above experiments would lead to the use of HW1 cells, perhaps after further *in vitro* manipulation, to investigate myelination *in vivo*. The animal work itself would be performed by collaborators in Cambridge using a rodent model of demyelination (Woodruff & Franklin 1999), although any prior manipulation of the cell line would be performed by the author.

Materials and Methods

Media Recipes - 10% FBS in DMEM

Dulbecco's Modifies Eagle Medium	(Gibco 41966-029)	500ml
Foetal Bovine Serum –heat inactivated	(Gibco 10108-165)	50ml
Penicillin / Streptomycin / Fungizone	(Gibco 15240-062)	5ml

Defrosting Cells from Liquid Nitrogen

10% Fetal Bovine Serum (FBS) in Dulbecco's modified Eagle Medium (DMEM) and a separate aliquot of FBS were warmed to 37°C in a water-bath. A 50% FBS solution was made by adding 4.5ml FBS to 5.5ml 10% FBS in DMEM in a sterile universal. The cryovial was removed from liquid nitrogen, defrosted in the waterbath and the contents transferred to a fresh 15ml centrifuge tube with a glass Pasteur pipette. 1ml of 50% FBS was added drop-wise to the 15 ml tube containing the cell suspension (to minimise sudden osmotic shifts). This was agitated gently, the remaining 9ml of 50% FBS was then added slowly and the resulting mixture centrifuged at 1500 rpm for 5 minutes. The supernatant was then removed, the cells were resuspended, and 5ml of fresh 10% FBS added. The cells were transferred to a T25 tissue culture flask (Nunc) and placed in the incubator with 5% CO_2 at the 33° or 39°C.

Passaging Cells

Phosphate buffered saline (PBS), 10% FBS in DMEM with 1% penicillin/streptomycin/Fungizone®(Amphotericin B) (PSF) and trypsin 1x (Sigma) solution were warmed in a 37°C waterbath. A T25 flask of sub-confluent cells was removed from the incubator, wiped with 70% alcohol and residual medium was aspirated off under the laminar flow hood. The cells were then rinsed with 10ml PBS before 50µl trypsin in 1ml fresh PBS was added to free the cells from the surface. The flask was incubated at 37°C until the cells rounded up and came off the plastic, and then tapped gently to facilitate dislodgement. 10 ml fresh 10% FBS was added to neutralise the trypsin and the resultant mixture was poured into a 15ml Falcon tube and centrifuged at 1200rpm (200g) for 5 min. The supernatant was removed, the cells resuspended, and 10ml fresh 10% FBS was added.

The cells were counted in a Neubauer haemocytometer with trypan blue exclusion.

1ml of the cell mixture was then added to 5ml fresh medium in a new T25 flask (1:5 split) and returned to the incubator

Cryopreserving Cells

Phosphate Buffered Saline, 10% FBS in DMEM with 1% PSF, Trypsin 1x solution and a separate aliquot of FBS were warmed in a 37°C waterbath. Freezing medium was then prepared by mixing 4ml FBS and 1 ml dimethylsulphoxide (DMSO) in a sterile Bijou tube. Cells were prepared as for passaging (see above) but resuspended in only 0.75 ml 10% FBS, triturated with 1ml Gilson pipette and split into two cryovials. 375µl pre-prepared freezing medium was added to each cryovial to make a final concentration of 50% FBS, 10% DMSO and 40% DMEM with 0.4% PSF. Cells were frozen in a "Mr Frosty" cryopreservation container, which was placed in a –70°C freezer overnight. The vials were transferred to the liquid nitrogen storage facility the next day.

Immunofluorescence

Cells in 100µl medium were seeded onto poly-lysine coated coverslips, for a final density when stained of about $5x10^3$-$5x10^4$cells per coverslip. The wells were flooded with medium after 1-24 hours and incubation continued until ready to stain. The coverslip was then removed from the well, washed once in culture medium containing 10% FBS and placed on a staining platform (humidified). (Cells that had been incubated in medium with less than 5% FBS required a blocking step using 5-10% FBS or Normal Goat Serum (NGS) for 1 hour to reduce background staining.) Test coverslips were then incubated with antibodies directed at surface antigens for 20 min at 37°C together with control slides that were processed identically but with the primary antibody omitted. They were washed twice in 10% FBS and incubated with appropriate secondary antibodies conjugated to fluorescent molecules for 20 min at 37°C. They were washed twice in 10% FBS again and fixed for 5 min in 4% paraformaldehyde. A further step involving permeabilization in ice-cold methanol (-20°C for 10 min) or 0.05% NP40 in PBS or 0.1% Triton X-100 in PBS for 5 min was added for intracellular antigens only. These cells were washed again and incubated with antibodies (to intracellular antigens) at 4°C overnight – or at 37°C for 20 min, followed by a wash and further incubation with appropriate secondaries (20min at 37°C). All slides were then washed a final time in 10% FBS, mounted in Vectasheild

(Vector laboratories) and sealed with nail varnish. Immunofluorescence was observed using an Olympus IX40 inverted microscope.

Bromodeoxyuridine Assay

Bromodeoxyuridine (BrDU) is incorporated into cellular DNA during the S-phase of the cell cycle, and thus labels cells which have been dividing while exposed. Cells harvested as above were plated out onto polylysine coated coverslips at low density (between 10^3 and $5x10^4$ cells per well – depending on the proliferative capacity of the cells, and the proposed incubation period) in 100µl culture medium and incubated overnight. The wells were then flooded with 400 µl culture medium and any required mitogens, and incubation continued in the appropriate experimental conditions. When ready to label, 0.5 µl stock BrDU solution (10mM – kept in aliquots at –20°C) was added and incubation continued for 1-24 hours (dependent on the estimated proliferative rate). BrDU incorporation was terminated by fixing cells with 2% PFA for 10 minutes at room temperature in the wells. This was aspirated afterwards. Cells were permeabilised with ice-cold methanol for 10 min at –20°C and then rehydrated with 10% FBS. DNA was denatured with 2N HCl for 30min at 37°C followed by two neutralisation steps using 0.1M borate buffer for 5 min at room temperature. The coverslips were then removed from the well, rinsed in DMEM(or PBS) with 10% FBS (or NGS) and placed on staining platform. They were incubated for 20 minutes with 6µg/ml anti BrDU in 10% FBS/DMEM, washed twice in DMEM/10% FBS and then incubated with fresh anti msIgG_1-FITC 1:100 and Hoechst 33258 (1:1000). Following a further wash step, they were mounted in Vectashield and sealed with nail varnish. They were viewed under the IX40 inverted immunofluorescence microscope with a high power objective lens. The number of cell nuclei staining for BrDU (green) was compared with those staining with Hoechst (blue) to give the BrDU index.

Methylthiazoletetrazolium Assay.

Methylthiazoletetrazolium (MTT) is reduced to a visible tetrazolium salt by sub-cellular organelles dependant on mitochondrial respiration. This reaction is proportional to viable cell mass, and can be used to measure cell numbers, cytotoxicity or proliferation (Mosmann 1983). A stock solution of MTT was made up in a culture hood to a final concentration of 5mg/ml. 100mg of MTT from a fresh bottle was added to 20ml of PBS (0.01M made with milli Q ultra pure water) then filtered through a

0.2μM filter. MTT is light sensitive, therefore the tube was covered with foil and stored at 4°C until required. 20μl of MTT was added to each well on the plate to be assayed using a multi-channel pipette and the plate incubated at 37°C for 1 hour. Using an aspirator and a 25 gauge needle, the media and MTT were carefully removed from each well. In a fume cupboard, 100μl of isopropanol was added to each well of the plate. The isopropanol dissolves the tetrazolium salt synthesised in the cells thus allowing a comparative reading to be made. The plate was left until the colour was dissolved (30 minutes). The plate reader was switched on and allowed to warm up for 30 minutes. The results were read on an Ascend plate reader with a measuring filter of 540nm, and a refractive filter of 620nm. Accompanying computer software was used to subtract the second value from the first, and subtract the chemical blank mean from the resultant means. Further calculations were performed using Microsoft Excel. Preliminary experiments determined the linearity of the assay (Figure 2-6).

Results

Five vials of HW1 were originally transferred from the laboratory in Cambridge to a liquid nitrogen freezer at Bristol. They were defrosted according to the protocol recorded in the methods section.

The first two cryovials were defrosted without any viable cells. Several possible explanations were considered. These included technical error, so minor modifications to the protocol were tried, based on suggestions from investigators in other departments. On the third occasion a very small number of cells (less than 0.1% of the total population), from a sample described as passage 8, survived to adhere to the tissue culture plastic. These cells survived the extremely low initial surface density and were expanded and subcultured to a working population. The very low yield from the frozen cells remained a concern. Had there been more cryovials available, this yield would not have been accepted and the cells would have been discarded. Further expansion allowed cells to be stored for later work. Viability from these samples, when defrosted after a week in liquid nitrogen, was in excess of 80%, although the protocol used (see methods) was apparently identical to that used to cryopreserve the original line. The reason for this discrepancy was not immediately apparent. After repeated enquiries, it finally transpired (some months later) that these cells had spent a considerable time at –80°C prior to transfer, a temperature not conducive to efficient cryopreservation.

Initial Characterisation

Morphology and Growth at Different Temperatures

Two flasks (approximately 10% confluent) from the same passage were placed simultaneously at 33 or 39°C and observed at 3 and 5 days.

Morphology

The cells assumed an elongated, slightly flattened appearance somewhat akin to fibroblasts (Figure 2-1). They had up to four short "processes", although it was unusual for these to be more than just extensions of the soma (NB these did not fulfil the description of processes as defined in Chapter 4). Secondary arborisations were almost never seen. The nuclei were large with prominent nucleoli. At 39°C the cells

remained similar in appearance at low surface densities (Figure 2-2), and never assumed the multiple ramified processes of oligodendrocytes or their later precursors. There was a tendency to adhere better to the uncoated tissue culture plastic at 39°, and this gave the cells a more flattened polygonal appearance(Figure 2-5). This became more obvious as the cells approached confluence, with progressive pavementing of the cells at 39°C, whereas their sister cells at the lower temperature retained the elongated shape and tended to grow over each other if left. Occasional cells at either temperature became very large and flat with a large nucleus. This was initially thought to be a transient stage, perhaps associated with cell division but these cells appeared to persist and no mitotic figures were visible.

Growth Characteristics

After the problems of the initial low cellular density following defrosting had been overcome, the cells grew rapidly at 33°C, with a 1:10 passage reaching confluence after about six days (estimated division time $24x6 / Log_2 10 = 43$ hours). Contrary to expectation, cells at 39°C continued to divide and became confluent slightly faster than at the lower temperature (on average at about 4-5 days). However, exact cell numbers were difficult to count near confluence, and the possibility remained that this was an artefact from their more flattened morphology occupying a larger surface area per cell.

This experiment was repeated several times with consistent results.

This suggested that the cell line was not behaving in the temperature sensitive manner originally described. Various possible explanations for this were considered:-

1) The experimental conditions were different

The cells were being exposed to a lower temperature that was allowing the oncogene to remain active, either because the incubator was not be maintaining its temperature or because the oncogene was being reactivated by the lower temperature when the cells were being examined.

2) The cell line had changed from its original description.

There had been a progressive alteration in the milieu of transcription factors and hence gene expression with a resultant change in the cellular phenotype and behaviour. (e.g. an intrinsic timer now signalled apoptosis upon oncogene inactivation, rather than maturation).

It had accumulated further copies of the oncogene, acquired unrelated mutations or had undergone a chromosomal rearrangement (aneuploidy).

3) The cells grown were not those of the original cell line described.

The cells had been contaminated with another cell line.

There had been an error in transit.

Many of these possibilities could apply merely to a subpopulation, or perhaps lead to a quantitative not qualitative alteration in response (e.g. the oncogene might require longer to switch off than originally described). Any subpopulation that matured or apoptosed amongst a rapidly growing population would be quickly swamped. Similarly any cell that acquired a survival advantage through genetic or epigenetic means could quickly dominate the culture.

The incubator temperature was carefully and repeatedly checked but was found to be accurately controlled.

The original experiments were repeated with the flasks left in the incubator throughout the experiment. This caused some problems, as the medium could not be changed. However there was still no evidence of growth arrest, or significant slowing at 39°, and the morphological features did not suggest differentiation. During all subsequent experiments, the design incorporated mechanisms for ensuring that removal from the incubator was eliminated or kept to a minimum, and that any medium added was warmed to the incubator temperature first.

The original investigator (who was now in New York) and Cambridge laboratory technical staff were contacted, and details about the experimental procedure discussed further. The possibility of a clerical error leading to the wrong cells being transported was also examined but rejected (all flasks were clearly labelled and dates tallied with that recorded in logbooks). However, this enquiry did uncover the error in transportation that had lead to the cells being exposed to –80°C. Further cells from the original liquid nitrogen store were defrosted and an acceptable yield (>60%) of viable cells was achieved from a cryovial labelled as passage 3. This was thought to be the earliest passage stored. However repeated experiments confirmed that these cells behaved indistinguishably from the first batch and failed to stop proliferating or show any morphological features of differentiation at the non-permissive temperature.

Immunofluorescence

Cells immortalised with a temperature sensitive oncogene have been described which subsequently escape from this temperature restriction. Some of these continue to proliferate at both temperatures while still retaining characteristics of the original parent cell and maintaining the ability to differentiate at the non-permissive temperature (Conejero et al. 1999). In spite of the absence of any morphological evidence of differentiation, it was decided to characterise the immunophenotype of these cells to identify what features had changed from when previously described. This work was started on cells from the first sample, but the experiments were repeated and continued on the earlier passage cells.

The experiments used A2B5, NG2 (Chondroitin Sulphate), O4, GalC (both Ranscht mAb & O1), GFAP (both poly and monoclonal) separately and in combination. Staining remained uniformly negative for all markers used. This was surprising. The original cell line was clearly positive for A2B5, NG2 and O4 even when growing at 33°C (Figure 2-4). The staining protocol appeared to be identical to that used in Cambridge. Repeated attempts were made with alternative protocols used by other investigators, but the cells were consistently negative. It was thought that technical inexperience was the most probable explanation for the failure to stain. Minor problems caused by lipid artefact and difficulties with live staining because of toxic preservatives in the antibody preparations were possible contributors. At this time, there were no opportunities for positive controls, as there were no other cell lines with these antigens available, and the primary glial work was still awaiting ethical approval. Two approaches to this problem were pursued. Firstly the original researcher requested access to the cell line and some samples (passaged and frozen at Bristol) were sent to New York, where they were grown up and stained. It was reported that the cells no longer stained for A2B5 or O4. Secondly, this researcher went to Cambridge to compare staining profiles using Cambridge reagents in parallel with reagents brought up from Bristol. The staining was done both by an experienced technician from Cambridge and this researcher, working in parallel. The Cambridge reagents demonstrated equivocal A2B5 staining, a result that was not seen using the Bristol reagents. None of the other antigens were detected.

Antimitotics

One further possibility that was pursued was that some of the cells did differentiate, but that these were rapidly overgrown by proliferative cells. To investigate this, cells were grown in the presence of anti-mitotic agents.

Firstly, the optimum dose to arrest cell growth was determined by plating out a fixed starting density (10^4 cells per well) in a 24 well plate, and making doubling dilutions of antimitotic agents, above and below the recommended dose. These were then incubated at 39°C. The dose and agent used permitted a significant number of cells to remain viable at one week, but inhibited proliferation. Cytosine Arabinofuranoside (AraC) at a dose of 1.25μM in isolation or a combination of 0.6μM 5-Fluorodeoxy Uridine (5-FU), 0.6μM AraC and 0.5μM Uridine (Antel 2003) both appeared to achieve the desired result. It was observed however that, if left, the majority of cells eventually died at any concentration of antimitotic agent sufficient to prevent them from reaching confluence. Since the agents used are incorporated into and disrupt the DNA of dividing cells – the implication was that the whole population remained mitotically active at 39°C.

Notwithstanding this conclusion, the experiment was continued and fresh cells were plated onto PLL coated coverslips, and left overnight to plate down. The wells were then flooded with medium containing the appropriate concentration of antimitotic agents and incubated at 33° or 39°C for 5 days without further manipulation. They were then photographed *in situ*, and immediately removed and stained for A2B5, GalC, GFAP, O1 and O4. The staining was negative for all antigens, but there was significant morphological change – most marked at 39°. The cells developed multiple extended processes, often with secondary, though rarely tertiary, arborisations. Although these were significantly different from their usual appearance, they bore little resemblance to oligodendrocytes or their precursors.

The possibility that inactivation of the oncogene leads to cell death was also considered. There is some support in the literature for this explanation (Jha et al. 1998). Human fibroblasts incorporating large T can replicate for some 20-30 generations more than their normal life span, but after this either senesce or die. Large T antigen appears to depend for its action on its inactivation of two tumour suppresser gene products – p53 and pRB. Inactivation of large T will lead to the cell reverting to its previous phenotype, and if it is beyond its natural life span, senescing or dying

(Hubbard-Smith et al. 1992). A very small proportion (10^{-5} – 10^{-8}) of fibroblasts incorporating large T do not senesce with the other cells, and those that survive this crisis progress to become truly immortalised. These cells presumably acquire an additional (genetic) event, which may involve stabilisation of telomere length. Even these cells depend on large T for continued proliferation (Radna et al. 1989;Wright et al. 1989).

Cells were grown at 33° or 39° in high or low serum in the presence or absence of antimitotic agents. After a week they were stained with a combination of Acridine Orange and Ethidium Bromide, which enables the identification of early and late apoptotic cells, and differentiates these from viable and necrotic cells (Leite et al. 1999). A few dying cells (both apoptotic and necrotic) were seen at both temperature in 10% FBS, but this figure was <2% for the cells grown at 39°C. These figures were similar in 1% FBS suggesting that this proportion of serum is sufficient for survival, but the proportion of dead or dying cells increased dramatically in the presence of the anti-mitotic agent AraC (Figure 2-4).

Proliferation

Three methods were employed to investigate the proliferative potential of these cells, and these studies were commenced at the point where faster proliferation at 39°C was first suspected.

The first method involved measuring the time taken for a flask passaged 1:10 to reach confluence. This was 6 (±2) days, but was unduly influenced by subtle differences in the protocol used. Cells at 39° reached confluence after 4-5 days; consistently earlier than those at 33°. However this technique suffered from several problems: It took no account of the time taken for the cells to settle and attach and calculations assumed logarithmic growth *ab initio*. Cells needed at least one medium change during this time, and it was very difficult to ensure that there was no drop in temperature during this time. The end point was hard to quantify, particularly because the cells at 39° pavemented quickly, whereas gaps remained between those at 33° until they were nearly peeling off. In addition subtle differences in starting density lead to certain areas reaching confluence before others, making the determination of a clear endpoint problematic.

The second method used BrDU, a synthetic nucleotide analogue, which is incorporated into cellular DNA during S-phase of the cell cycle. Any cell that is

passing through the S phase during BrDU exposure can then be labelled with an antibody directed against BrDU, which can then be visualised with a fluorescent secondary. The BrDU index can then be ascertained by the proportion of labelled cells divided by total cells) to be obtained. In practice, this procedure was rather variable. Cells at 39° stained very strongly, and it was very easy to distinguish labelled cells from unlabelled cells, and thus to obtain an index. At 33° however, cells did stain, but much less brightly, and the difference between labelled and unlabelled cells was less distinct, increasing the potential for error. In addition, the Hoechst stain appeared to interfere with the anti-BrDU (Hoechst binds to double stranded DNA, anti-BrDU will only label partially denatured (single stranded) DNA (Molecular Probes 2001)), so nuclei had to be counted under phase contrast. This was acceptable at low starting densities, but became problematic as the cells reached confluence. No reliable qualitative comparison was possible but in excess of 95% of HW1 cells incorporated BrDU at 39°C after a 36-hour pulse (Figure 2-5).

The third, and most reproducible method, relies on the property of viable cells to reduce MTT to a dark blue formazan dye. Even recently killed cells fail to change the dye – enabling it to be used as a measure of cell number (Mosmann 1983). This reaction was originally presumed to occur in mitochondria, but recent work suggests that the reaction itself takes place in other organelles (Morgan 1998) fuelled by mitochondrially derived NADH. Prior to using this assay, the linearity of the assay was confirmed by comparing doubling dilutions of a cell numbers with the MTT readout after allowing the cells to adhere for 2 hours (Figure 2-6).

Three experiments using the MTT assay were performed. These looked at proliferation against starting cell number, temperature (Figure 2-9), serum concentration (Figure 2-9) and a selection of proliferative cytokines implicated in glial growth and maturation (Figure 2-10). The cells showed a characteristic growth curve – "lag", "log" and "plateau" phases were all identified. The previous observations were confirmed – HW1 cells grow faster at 39°C as measured by the gradient of the growth curves, and there was no evidence of slowing except as the cells reached confluence. Interestingly, the cells appeared to stop growing at a lower final surface density (maximum MTT value) when grown at 39°C compared with cells at 33°C (Figure 2-7 &Figure 2-8). This is in keeping with the previous observation that the cells at 39°C are more flattened, adhere better and become confluent without overlapping. Cells grew faster in 10% serum than in 1%, an effect that could not be restored by the

addition of EGF, FGF, PDGF or both F&PDGF to the lower serum concentration, although there was a modest increase in MTT value after the addition of both FGF & PDGF (Figure 2-10).

The key observation in all these experiments was that growth was persistently *faster* at 39° than at 33°. This was not dependent on cell density, although there was evidence that the effects of contact inhibition at 39° caused growth arrest earlier than at 33°. There was also little evidence of growth enhancement by any of the growth factors used. Growth appeared to be maximal at low density, although below a certain threshold density, cell survival diminished.

Discussions

HW1 was originally designed to facilitate investigation of the human oligodendrocyte lineage. The key attributes (both intended and reported) were that it was phenotypically similar to the human oligodendrocyte progenitor when grown at 33°C, a temperature at which the transforming oncogene was active, and was able to proliferate rapidly under these conditions. When grown at the non-permissive temperature it was reported to stop proliferating and start expressing antigens suggestive of progressive glial differentiation. Furthermore at the non-permissive temperature it was reported to be bipotential, able to differentiate along the oligodendrocyte lineage in low serum conditions, or to express markers of type 2 astrocytes when grown in 15% FBS or above, a characteristic of the rodent oligodendrocyte progenitor.

The following conclusions were made from the experiments described above:-

- The cell line does not have the same immunophenotype as originally described at 33°C.
- It does not appear to express demonstrable amounts of any of the glial progenitor antigens tested for at 33°C.
- The cell line neither slows down nor stops proliferating at the non-permissive temperature (39°C).
- The cell line does not show any immunological or morphological evidence of differentiation at 39°.
- The lack of differentiation and the failure to stop proliferating could not be attributed to unsustained temperatures alone.
- There is no evidence to support the hypothesis that a subpopulation of these cells behaves as originally described.
- There is no evidence to suggest that a significant proportion of these cells is undergoing apoptosis at 39°.

This left two likely explanations for the observed results; either the cell line had changed since it was first described, or that the cells under investigation were not HW1.

The cellular and antigenic phenotype is dependent both on the pattern of gene expression within the cell, which in turn is controlled by transcription factors within the nucleus, and the genome itself. It is reasonable to speculate that changes in gene

expression occurring over a limited number of generations, might be restricted enough for the cell to retain some demonstrable antigenic similarities to the parent cell. Thus the absence of any such marker, while not excluding a change merely in gene expression, might suggest a more fundamental change.

As previously discussed, many SV40 immortalised cell lines experience a period of crisis – where the majority of cells senesce, and a small population, presumably with further somatic mutations, continue growing and eventually overrun the original cells. There was no description of a period of crisis occurring to this cell line, which was investigated while still in its infancy. The poor survival from the defrosting process is not likely to have been due to a crisis phenomenon, in that sub-culturing well beyond this passage number had occurred in Cambridge without evidence of this. However, selection out of cells containing mutations conferring a degree of resilience to the process of defrosting, could provide a mechanism for a fundamental change in genotype.

The acquisition of further copies of tsTag may be responsible for altered behaviour of cell lines immortalised in this manner. The use of a replication defective virus as a vector, the original determination of a single integration site and the magnitude of the difference in phenotype of this cell from that described mitigates against this explanation.

Large T antigen is known to be both clastogenic and aneuploidogenic (Ray et al. 1992), and this action may be exacerbated by its alteration of apoptotic mechanisms. The high frequency of chromosomal abnormalities in certain SV40 induced cell lines suggests that it may play a role in the immortalisation process in these cases. In addition, there is some evidence that cryopreserving cells in liquid nitrogen may also lead to chromosomal breakage – although it appears that affected cells often do not survive.

It was thus decided to proceed to formal karyotype analysis. This was performed by the regional cytogenetics laboratory at Southmead hospital on a live culture of passage 5 cells.

Karyotype Analysis of HW1

The report returned as follows:-

"Metaphases obtained from cell culture were apparently of rodent origin. No evidence of human metaphases seen." [*Dr T Davies BSc PhD FRCPath Cert MHS*]

After further questioning the suggestion was made that they were most likely of rat origin, although the exact species was not determined with security (Figure 2-11). Contamination between mammalian cell lines is a well-recognised hazard of cell culture experiments, particularly when different cell lines are being used in the same laminar flow cabinet. The uniform behaviour of the cell line suggests that the cells were contaminated prior to their arrival in Bristol. In addition, no rat cell work was proceeding or had been undertaken in this laboratory prior to our arrival, and the laboratory had been empty for in excess of a year. The only rodent cells used in the laboratory at the time of these experiments were two mouse hybridoma cell lines – and they grow in suspension, rather than as an adherent monolayer.

Attempts were made to source cells prior to the contamination. Thus cells as early as passage three were retrieved from Cambridge and analysed further. Attempts were also made to grow these in limiting dilutions and with mitotic agents, but all cells retained the characteristics described. A further passage 3 vial was defrosted and immediately sent for karyotyping – all cells were of rodent origin.

Interestingly, one other cell line, named HW2, had been produced in Cambridge. This had been less promising as glial antigens, though apparently present, were less consistently identifiable. The earliest passage of these cells were karyotyped as well and identified as of rodent origin also. It seems likely therefore that the cells were contaminated either at the time of transfection, or shortly afterwards. The most likely contenders were the RAT 1 cells used to assess infectivity prior to infection of the glial prep. PA317 cells, a mouse embryo line with herpes, were used as packaging cells and would be another contender. Although it should be possible to identify whether the cell behaved like either of these two, this was considered marginal to the aims of the thesis, and resources were limited.

The rationale for using transformed cell lines remains sound, but as has been demonstrated, this approach has not so far been fruitful. The facilities for transforming cells are not currently available in the Glial Cell Laboratory here in Bristol and so no further attempts were made in this direction. While this approach may again gain credence, recent advances in stem cell biology and methodological improvements in primary oligodendrocyte culture may reduce the need for such lines in the particular realm of human oligodendrocyte biology.

Illustrations

Figure 2-1 HW1 Growing at 33°C

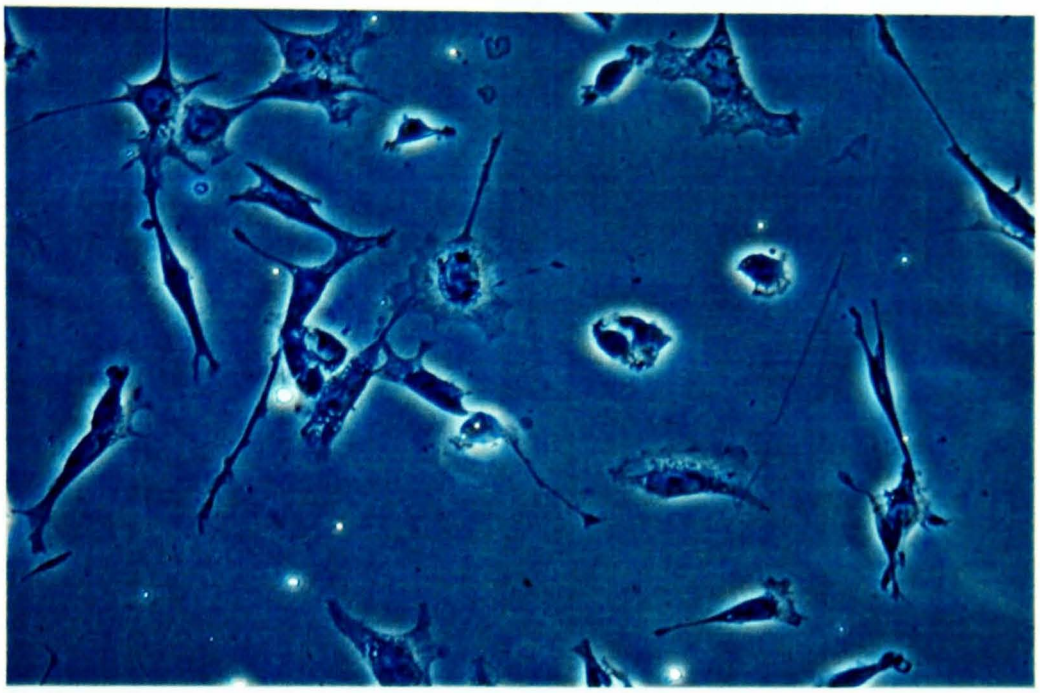


Figure 2-2 HW1 Growing at 39°C

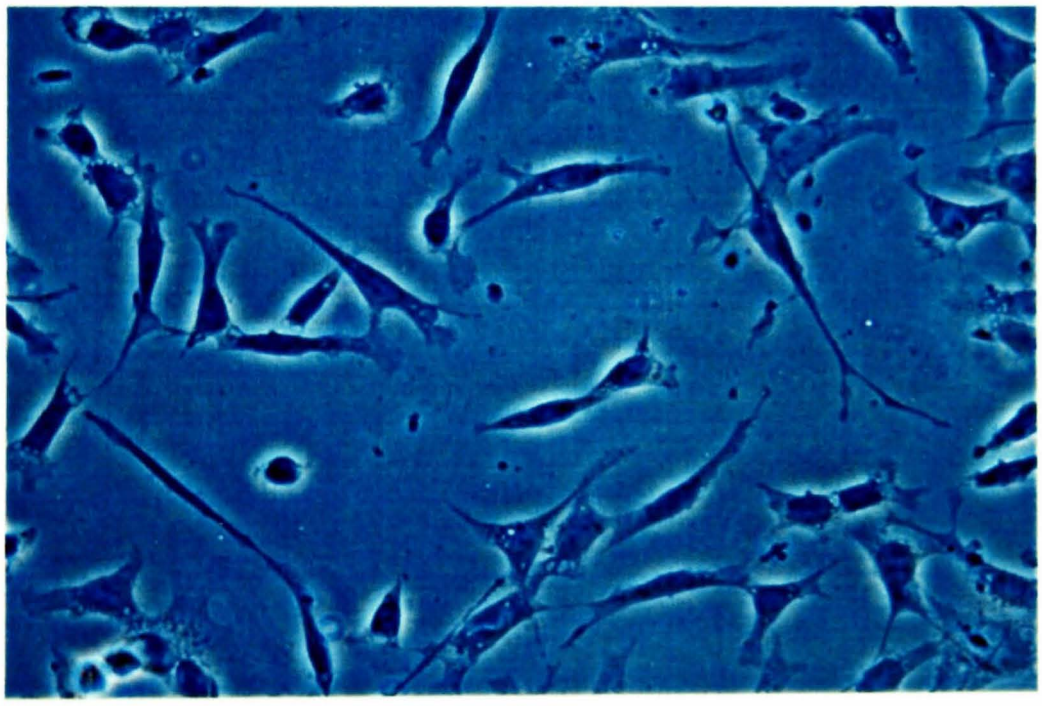


Figure 2-3 HW1 as Originally Reported

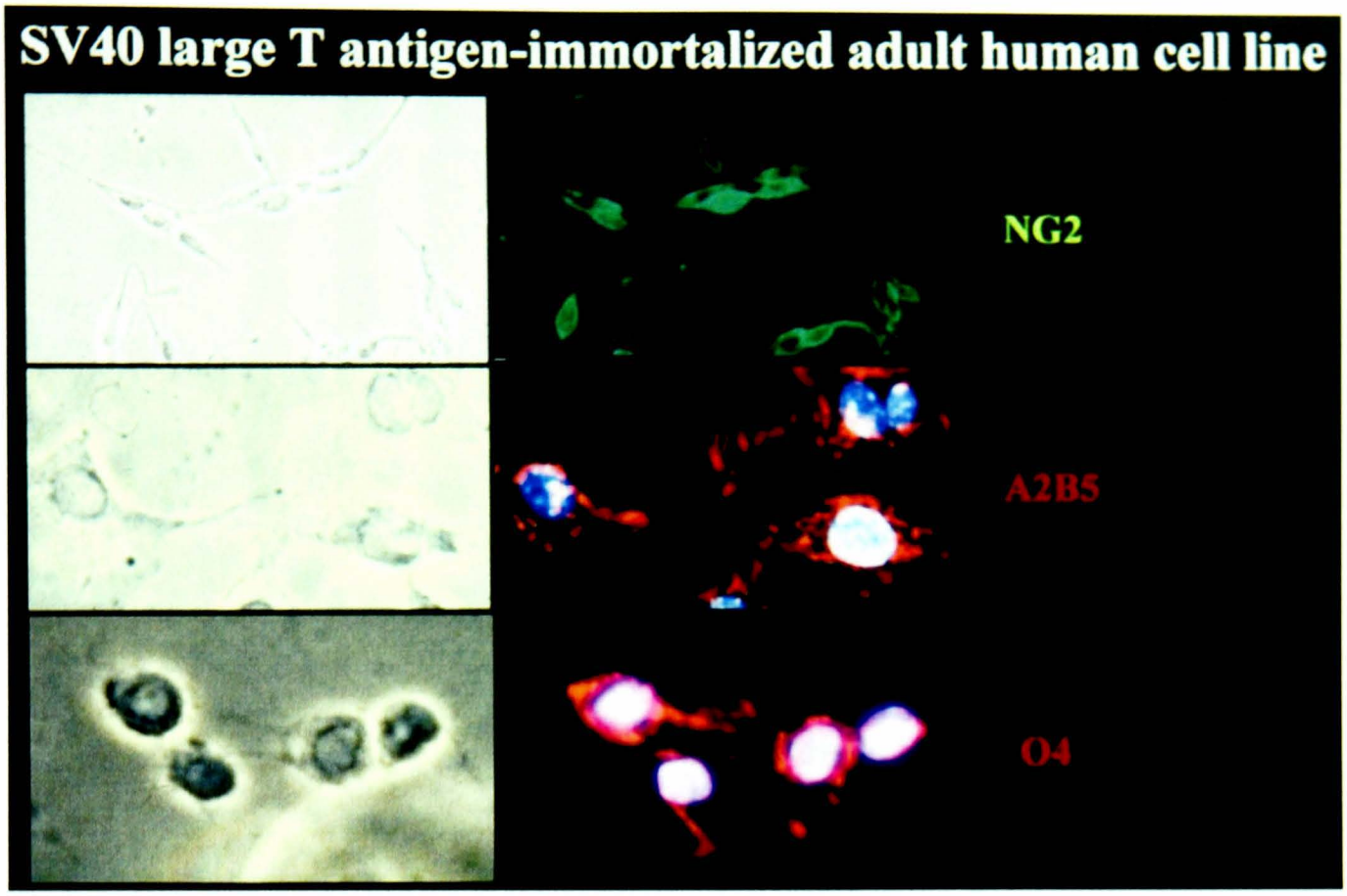


Figure 2-4 Apoptosis of HW1 Under Different Growing Conditions

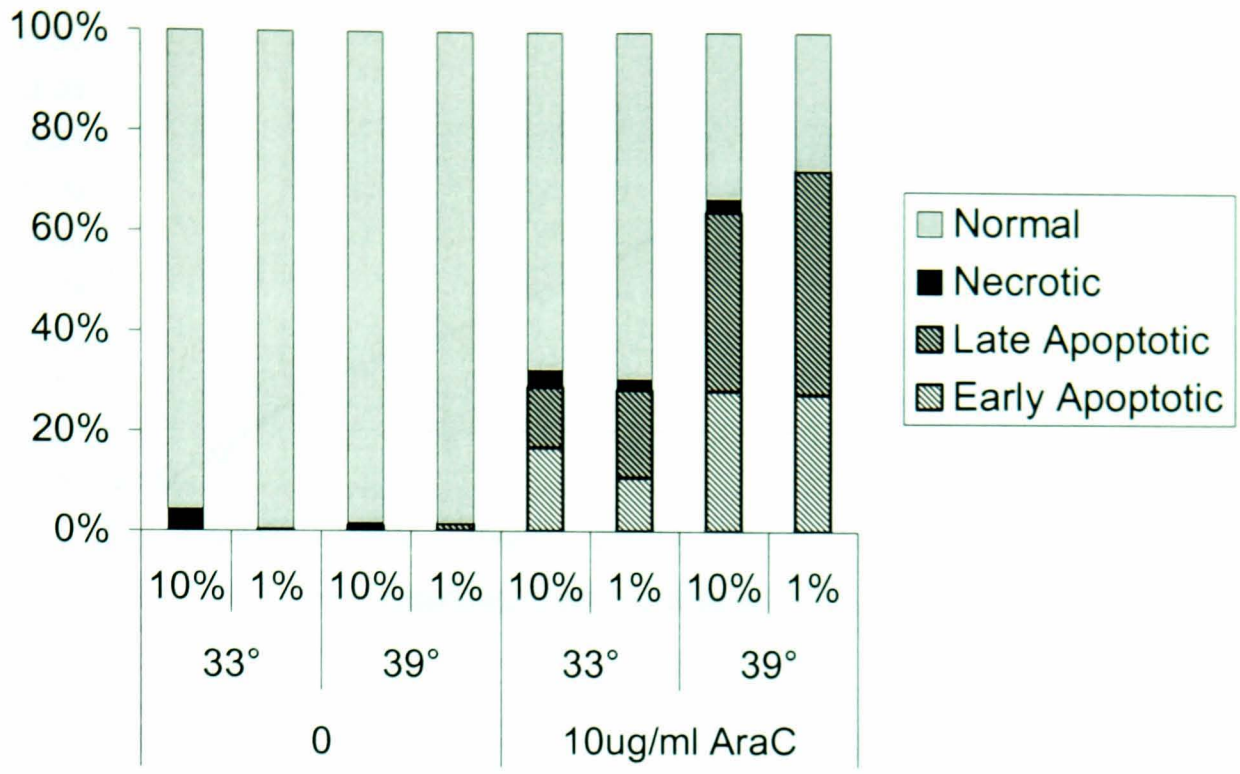


Figure 2-5 HW1 Growing at 39°C Incorporates BrDU after a 36 hour pulse (Combined BrDU-FITC and phase contrast x200)

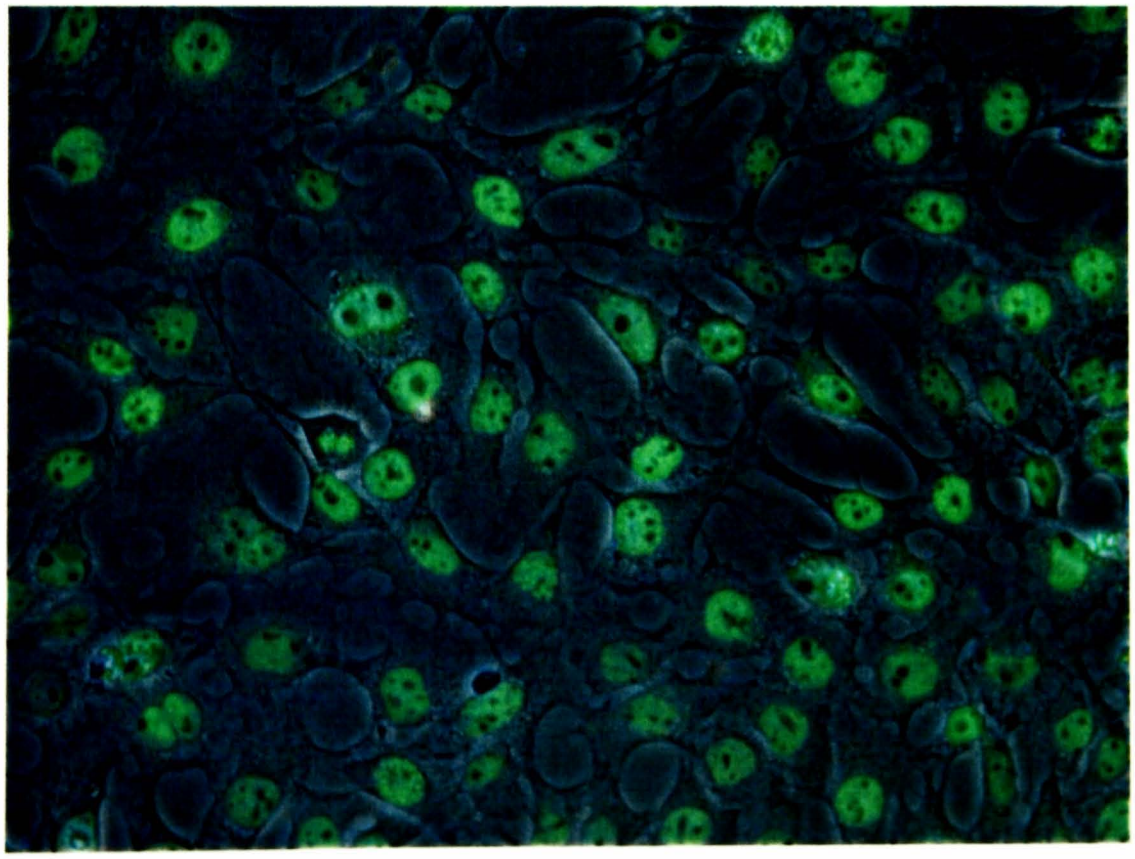


Figure 2-6 Linearity of the MTT Assay as a Measure of Starting Cell Numbers

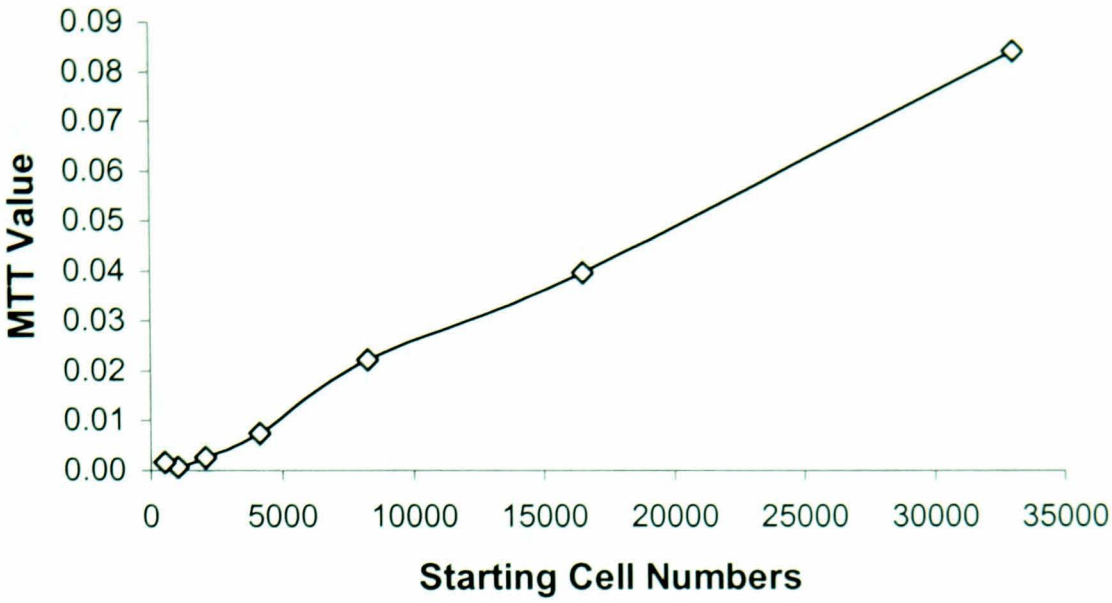


Figure 2-7 Growth Curves for HW1 for a Range of Seeding Densities at 33°C

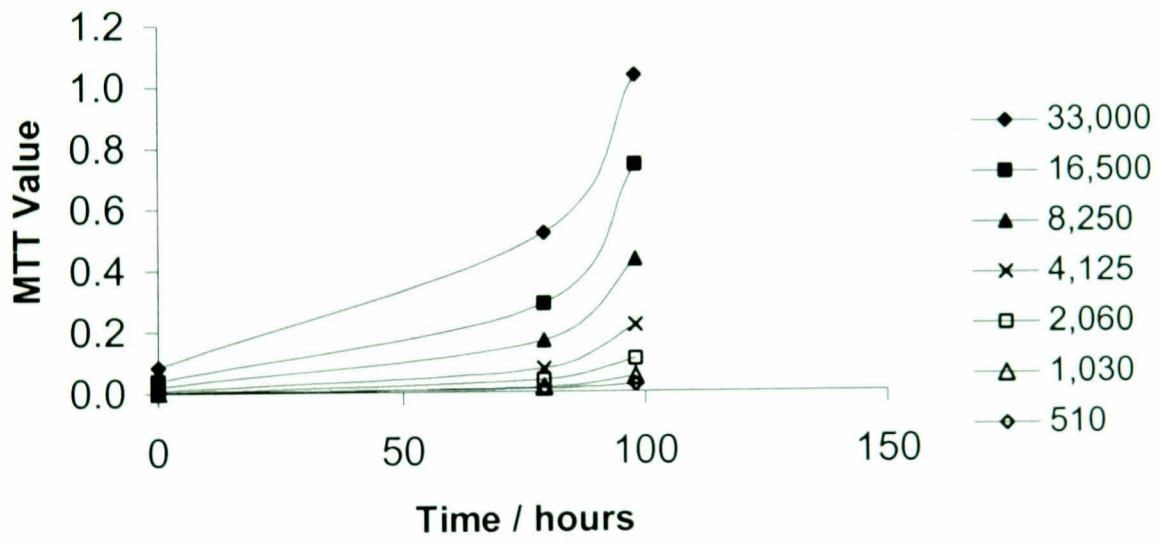


Figure 2-8 Growth Curves for HW1 for a Range of Seeding Densities at 39°C

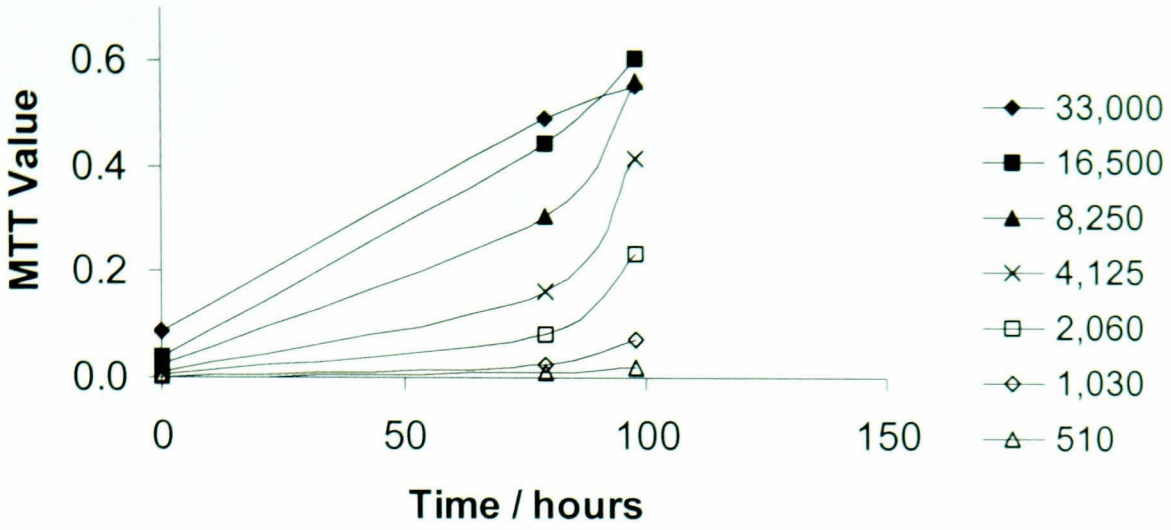


Figure 2-9 The Effect of Temperature and Serum Concentration on HW1

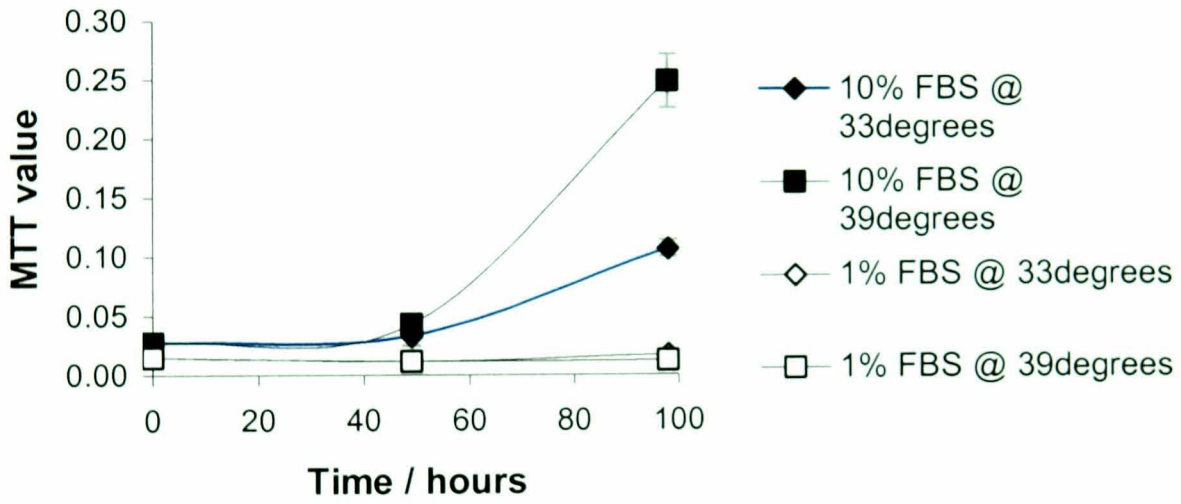


Figure 2-10 The Effect of Growth Factors on HW1 at 39° in 1% Serum.

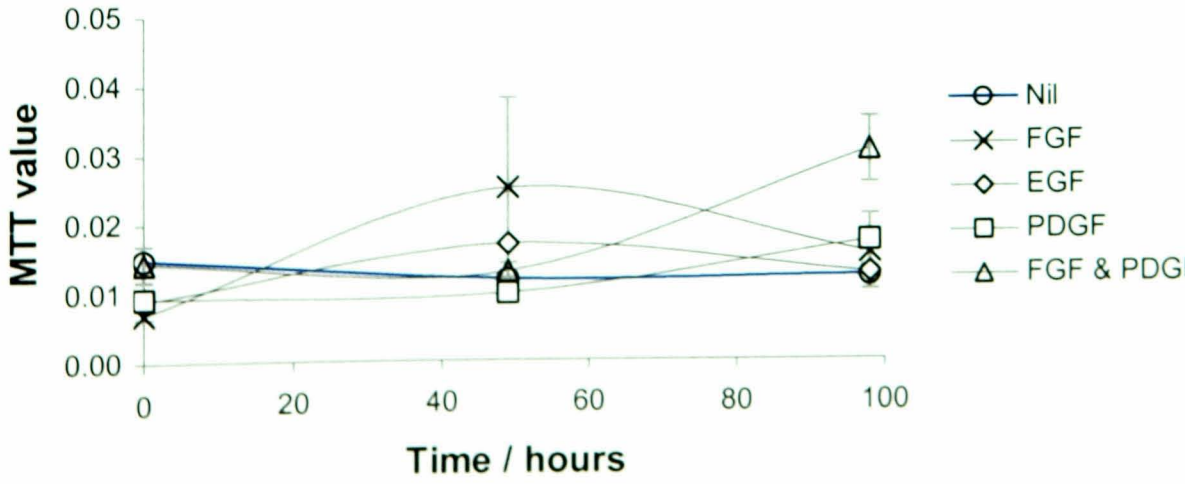
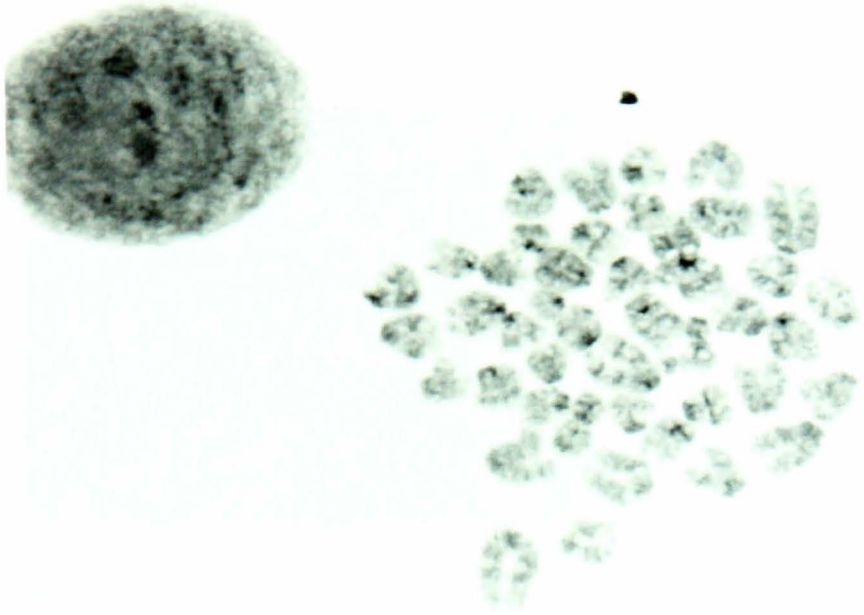


Figure 2-11 Metaphases from HW1 show typical rodent chromosomes



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Illustrations

Figure 3-1 CG4 cells growing in B104 conditioned medium (x 200)

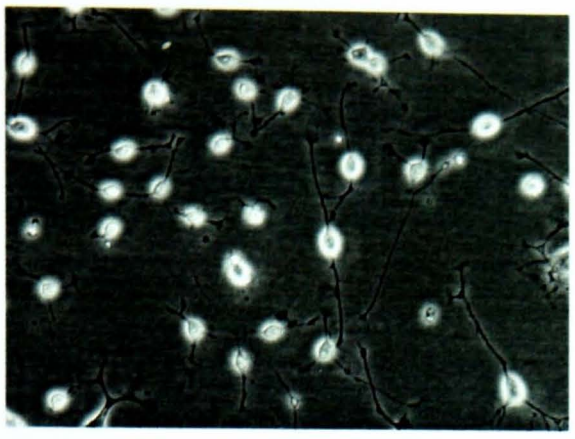


Figure 3-2 After four days in PDGFAA alone, CG4 cells show signs of differentiation. (x 400)

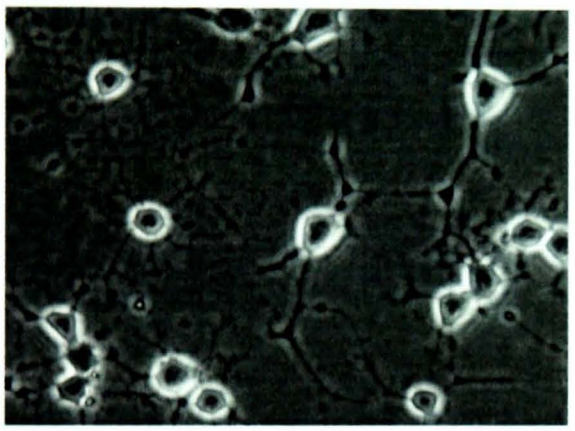


Figure 3-3 When cells grown in PDGFAA are placed in 15% FCS they flatten and adhere, and start to express the astrocytic marker GFAP (not shown). (x 200)

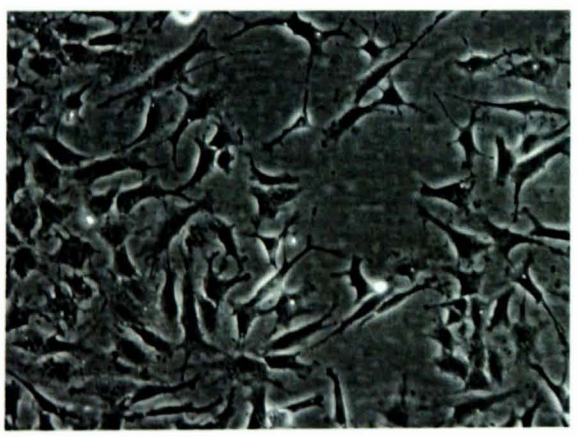


Figure 3-4 The changes seen in Fig. 3 are reversed by the removal of FBS and addition of FGF (x 200)

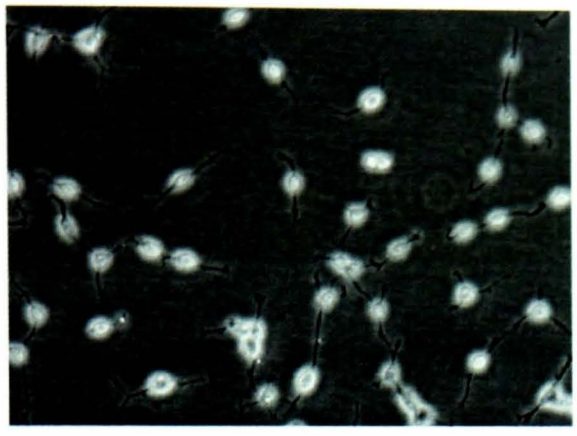


Figure 3-5 Cells grown in FGF alone adhere poorly to uncoated tissue culture plastic and form clumps, but further cell division is limited and true sphere formation is not seen. (x 200)

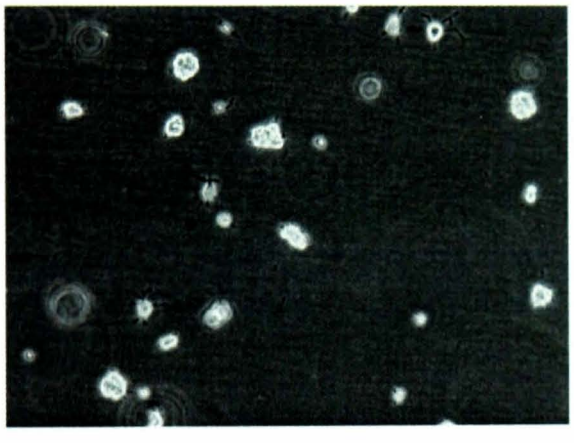


Figure 3-6 Summary of protocol and results published by Kondo and Raff.

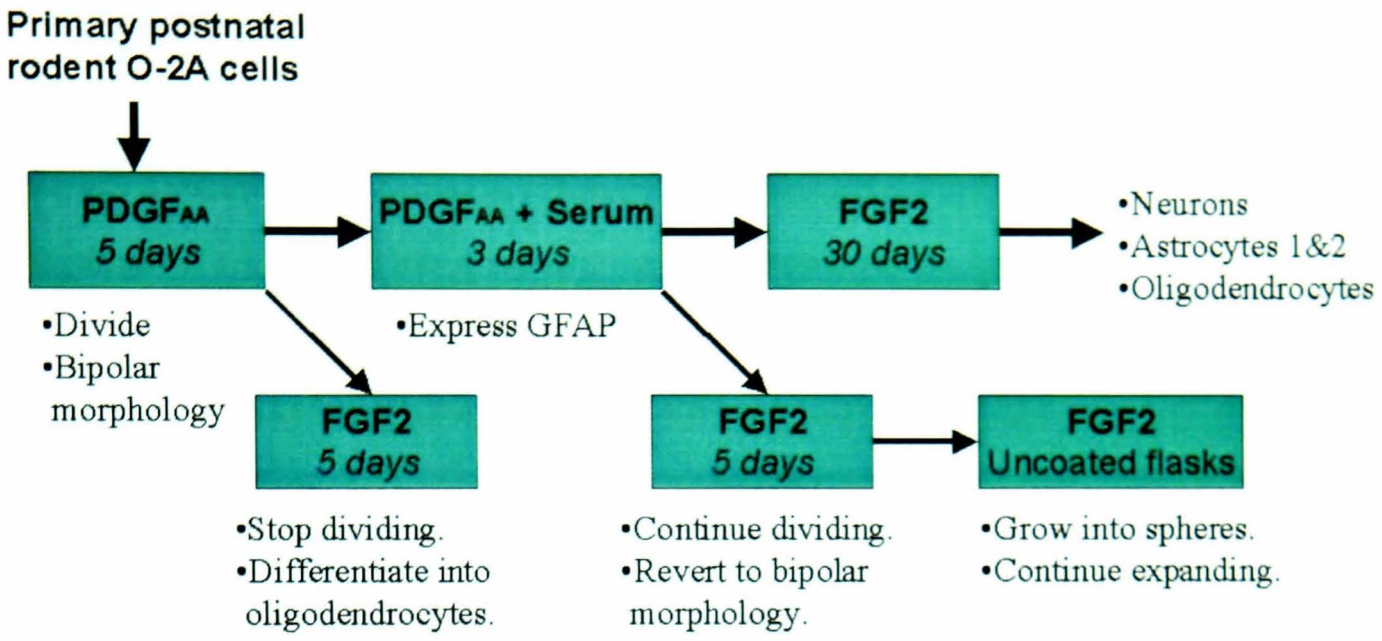
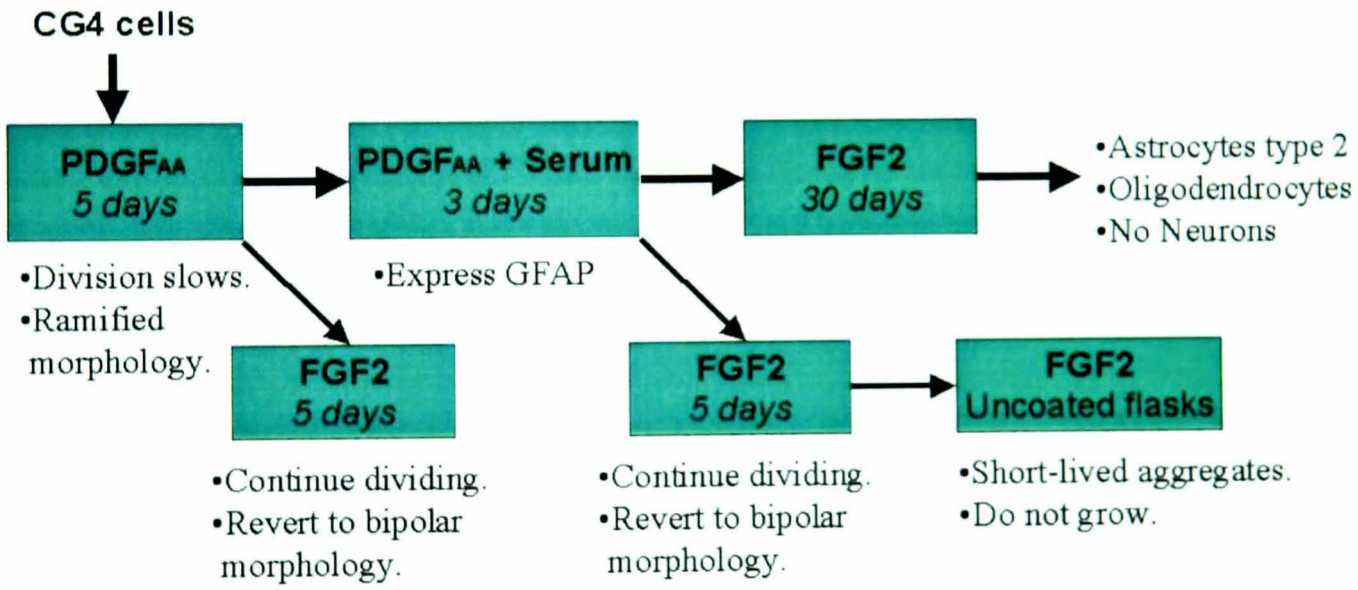


Figure 3-7 Summary of results obtained with CG4 cells.



Chapter 4. Identification, Isolation and Mitogenic Expansion of Human Oligodendrocyte Progenitors from Adult Human Brain

Introduction

Part 1 Identification of Human Oligodendrocyte Progenitors

Regardless of the source of progenitors that might be used in any putative remyelination therapy, adult CNS tissue, fetal tissue, mesenchymal cells etc, robust and reliable means of identifying progenitors must be available. Likewise, a prerequisite to the study of progenitor biology in MS lesions and normal tissue is the ability to identify these cells.

The rodent oligodendrocyte progenitor was first identified *in vitro* in perinatal rat optic nerve cultures (Raff et al. 1983) and subsequently in the adult rat CNS (Ffrench-Constant & Raff 1986) using the monoclonal antibody A2B5 (Raff et al. 1983). A2B5 was subsequently shown similarly to act as a specific label for oligodendrocyte progenitors cultured from other parts of the developing rodent CNS (Bottenstein et al. 1988), from the adult rodent CNS (Baas & Barnstable 1998;Wolswijk & Noble 1989), and from the developing human optic nerve (Kennedy & Fok-Seang 1986) and adult human brain (Scolding et al. 1995). The other cell type that expresses A2B5 antigens in both rodent and human mixed glial cultures, the so-called type 2 astrocyte, can be clearly discriminated both by its characteristic morphology and its co-expression of GFAP (Raff et al. 1983).

The oligodendrocyte progenitor has, however, proved very difficult to study *in vivo*. The A2B5 antibody (which is directed against specific tetragangliosides) also stains neurones in tissue sections (Fredman et al. 1984;Majocha et al. 1989) and cannot be used to identify progenitors. Another anti-ganglioside monoclonal antibody thought to be specific for the oligodendrocyte progenitor, LB1 (specific for G_{D3}), also binds to microglia (Wolswijk 1994;Wolswijk 1995), while a third marker, the PDGF-α receptor (Hart et al. 1989;Pringle et al. 1992), has been shown also to be expressed by neuronal sub-populations (Vignais et al. 1995).

Antibodies directed against a chondroitin sulphate proteoglycan, the NG2-antigen, have been shown to label neonatal and developing rat oligodendrocyte progenitors *in vitro* and in tissue sections (Nishiyama et al. 1996a;Ong & Levine 1999;Pekny et al.

1995). Recently investigators have used this antibody to identify adult human oligodendrocyte progenitors in tissue sections (Chang et al. 2000a;Chang et al. 2002). Furthermore, their results suggest that cells bearing this antigen are very numerous, an observation in conflict with some earlier estimates of human oligodendrocyte progenitor populations (Maeda et al. 2001;Scolding et al. 1998)and estimates of adult rodent progenitor numbers.

The adult rodent "O2A" progenitor has a number of morphological, immunocytochemical and behavioural differences from its neonatal counterpart (Ffrench-Constant & Raff 1986;Wolswijk et al. 1991a;Wolswijk & Noble 1989), while the immunocytochemical (and proliferative) characteristics of the adult human oligodendrocyte progenitor also differ from rodent cells (Armstrong et al. 1992;Ffrench-Constant & Raff 1986;Scolding et al. 1995;Wolswijk & Noble 1989). Therefore, extrapolating the finding that NG2 is a marker of the developing rodent oligodendrocyte progenitor to adult human glia must depend on direct experimental support.

It was decided to address the labelling characteristics of NG2 in adult human glia *in vitro*, where individual cells can be separately identified. To do this requires a "gold standard" with which NG2 can be compared. A2B5, a monoclonal antibody directed predominantly against epitopes on the ganglioside GQ (Eisenbarth et al. 1979), is an appealing candidate. There is considerable experience in its use in vitro, indeed it was used in the earliest descriptions of the oligodendrocyte progenitor (Raff et al. 1983). Cells labelling with this antigen in mixed human glial preparations have similar phenotype and differentiation potential to rodent cells (Scolding 1998;Scolding et al. 1995). Cells isolated from adult humans on the basis of early activity of oligodendrocyte-specific promoter activity express A2B5 (Roy et al. 1999) Furthermore, cells sorted on the basis of this antigen from adult human white matter develop into oligodendrocytes following transplantation (Windrem et al. 2002). The considerable problems associated with its use in tissue sections, caused by cross-reactivity with a neuronal population, are not relevant to unexpanded mixed glial cultures from adults - neurons do not survive the dissociation protocol (Antel 2003). The development of the type 2 astrocyte phenotype in culture necessitates co-staining with both A2B5 and GFAP to differentiate these with security from the undifferentiated oligodendrocyte progenitors. This limits the number of other antigens that can be interrogated on a single slide, but does not detract from its specificity when

this factor is taken into consideration. The identification of a new marker for oligodendrocyte progenitors that avoids the need for this second antigen would be a significant advance.

Preliminary findings (made by previous researchers in this group) suggested that two separate NG2 antibodies reliably label adult human oligodendrocyte progenitors in cell cultures, but also bind to GFAP-positive astrocytes and possibly microglia. The hypothesis that NG2 is a specific and sensitive marker of adult human oligodendrocyte progenitors *in vitro* was tested against A2B5 as an established standard. Quantitative assessment was performed in primary cultures obtained from adult human surgical specimens. The sensitivity and specificity of this antigen as a marker of these cells was determined, and other populations that can express the NG2 antigen in these circumstances were identified.

Part 2 Isolation and Expansion of Human Oligodendrocyte Progenitors

Closely related to issues of identification is the problem of isolating substantial numbers of progenitors from the mixed glial preparations. A major impediment to the study of the human oligodendrocyte lineage has been the difficulty of obtaining sufficient numbers of purified cells for experimentation.

In rodents this problem was solved some time ago. Neonatal rodent progenitors are highly proliferative cells and can be induced to undergo substantial expansion *in vitro* using FGF2 and PDGF$_{AA}$ in combination (Louis et al. 1992;McKinnon et al. 1990;Noble et al. 1988;Raff et al. 1988). Thus a purification process that yields only small numbers of cells can be combined with a simple but effective amplification step to yield a substantial population for further study. Furthermore, there are few limitations in the provision of source tissue – if necessary, tissue from several genetically related age-matched animals can be combined.

However, sources of human tissue are scarce and often erratic. While human progenitors have been shown to undergo DNA synthesis on an astrocyte monolayer, this was not reproducible using defined media and recombinant mitogens (Scolding et al. 1995). Furthermore, the astrocyte monolayer, while able to support limited division, failed to produce substantial numbers of these cells, as would be required for a thorough investigation of their biology. Any purification step would need to be

highly efficient to provide a reasonable yield from this method, and substantial improvements to the amplification steps are urgently required.

Various approaches to the purification problem have been tried. Rodent populations can be purified by means of differential adhesion to various substrates – and by shaking loosely adherent cells off a mixed monolayer (McCarthy & de Vellis 1980). However, this is inefficient, relies on a large population of starting cells and further substantial proliferation in mixed culture, and one might anticipate the need for several experiments to optimise this in a new species. This luxury is not available with such limited human material. A second option that has the added benefit of improved population purity is to use sequential immuno-panning (Stallcup & Beasley 1987) using the A2B5 antibody, but this too needs large volumes of starting material.

Flow activated cell sorting is in widespread use in haematology laboratories and two approaches have been used to purify human glial preparation with it. The simplest option is to label cells with a directly conjugated fluorescent antibody as a marker – although for most glial antigens these may not be commercially available and a secondary antibody would probably be required. The second option is to transfect the gene for green (or blue (Martinez-Serrano et al. 2000)) fluorescent protein into target cells under control of a cell-type specific promoter, as has successfully been performed by Roy et al. (Roy et al. 1999). How the basic biology of the cell might change as a result of this genetic manipulation is unclear.

One elegant option that avoids the need for genetic manipulation is to use immunomagnetic separation (Wright et al. 1997). This simple method, which relies on magnetic beads coated with antibody to bind to, and pull out, specific cell types based on surface antigen expression, is both efficient and versatile. Two kits, which differ in the bead size and separation method, are available commercially. These techniques are gaining credence both in experimental paradigms, and for clinical purposes.

Furthermore, in the final months of experimentation, a protocol for selection of adult human oligodendrocyte progenitors on the basis of A2B5 antigen expression was published. The benefits of this technique were reported by the same authors who had previously used the more complex promoter-defined separation method (Roy et al. 1999;Windrem et al. 2002).

This method was tested as a viable means of purifying progenitors and the cellular yield was assessed antigenically. The purity achieved with this method provided

important insights into antigenic expression that are not easily demonstrated using mixed glial preparations alone.

Part 3 The Proliferative Requirements Of Human Oligodendrocyte Progenitors

The third component of these experiments was that of optimising the growth conditions for human oligodendrocyte progenitors. Some of this work was done in parallel with the above studies to maximise the use of available tissue. Substantial, but controlled, proliferation and *in vitro* expansion of adult human progenitors remains an important goal of remyelination biology and is as yet unrealised. However, these cells have been isolated, grown *in vitro* for prolonged periods and even shown to undergo limited proliferation (Scolding et al. 1995), so many of the basic requirements for their growth are already known. There have been important advances in the media requirements of closely related cells, which might reasonably be extrapolated to adult human oligodendrocyte progenitors (Arsenijevic et al. 2001;Carpenter et al. 1999;Pagano et al. 2000;Zhang et al. 2000). These include the use of high glucose media and of DMEM/F12 based recipes rather than N2B3 (used in earlier attempts), which has subtly different hormonal and micronutrient concentrations. Preliminary experiments demonstrated improved yields with these changes but no evidence of cell division in the progenitor population (data not shown).

The working hypothesis remains that human progenitors will divide *in vitro* in response to mitogens identified from rodent work, and as such can provide a source of human progenitors for experimental and therapeutic purposes. Earlier failures to stimulate adult human oligodendrocyte progenitors using FGF2 and PDGF$_{AA}$ do not disprove this hypothesis. The absence of demonstrable proliferation with these two may be attributable to reasons other than the differing mitogen requirement of human cells. Classical tissue culture teaching suggests that sufficient surface density can be critical to *in vitro* growth (Antel 2003). Furthermore, the contributions, both positive and negative, from other cell types in mixed cultures are hard to quantify, and the presence of anti-proliferative signals might explain their reluctance to divide. Thus attempting to grow purified progenitors at high density was an important priority of these experiments.

Our understanding of mitogen requirements, summarised in the general introduction, suggests that PDGF$_{AA}$, FGF2, NT-3 and stimulation of the IGF receptor with high

insulin concentrations are likely contenders for any recipe (Baron et al. 2000;Engel & Wolswijk 1996;McKinnon et al. 1990;McKinnon et al. 1993;Wolswijk & Noble 1992). The appreciation of the role that extracellular matrix ligands have to play in this process suggests that vitronectin may be important, at least with more physiological concentrations of these mitogens (Baron et al. 2002). These conditions were therefore explored alongside the experiments to determine cell identity. They provide important evidence that adult human progenitors can be grown *in vitro* in defined media, and suggest that the mitogens predicted by rodent studies may indeed be relevant to human studies.

Materials and Methods

Adult human glial cultures

Cells of the human oligodendrocyte lineage were cultured using modifications of a protocol described elsewhere (Scolding et al. 1995)from normal-appearing white matter removed at the time of anterior temporal lobe resection for intractable epilepsy. (Previous studies confirmed that comparable tissue, dissected from sites distant from the epileptic focus, was histologically normal (Scolding et al. 1995)). On one occasion cerebellar tissue resected during the removal of a benign acoustic neuroma was used, as indicated in the text.

All patients gave their consent for resected tissue to be used for research purposes, and ethics approval was obtained from both local and regional ethics committees. Tissue was transported to the laboratory at 4°C in Hibernation medium (Hibernate™ A, BrainBits, Southern Illinois University) supplemented with penicillin / streptomycin / fungizone 1% (Invitrogen). Tissue was processed between 1 and 12 hours post resection.

Dissociation

Tissue was placed under a tissue culture hood, and meninges and large blood vessels were dissected off. The tissue was then minced with a scalpel and digested. Tissue was incubated at 37°C for 20 minutes in 5 ml Enzyme solution 1 (containing MEM (Sigma M7278), 0.116mg/ml trypsin (Sigma T4424), 0.184mg/ml collagenase III (Lorne LS 4208) & 18.4μg/ml $DNAseI_{IV}$ (Sigma D5025)). The mixture was then centrifuged at 200g for 5 minutes, the supernatant replaced with a further 5ml enzyme solution 1 and incubated for a further 20minutes. Following centrifugation the supernatant was then replaced with 5ml enzyme solution 2 (containing MEM, trypsin-EDTA (Sigma T3924) 23 & 9.2μg/ml respectively, 0.184mg/ml collagenase III & 18.4μg/ml $DNAseI_{IV}$) for 15 minutes at 37°C and centrifuged. Finally the supernatant was replaced with 1ml of enzyme solution 3 (containing MEM, 2.4mg/ml trypsin inhibitor (Sigma T6522), 1.9 μg/ml $DNAseI_{IV}$, 1.6mg/ml bovine serum albumin (Sigma A4919) and 5% heat inactivated fetal bovine serum (Sigma F4135)) and triturated three times through 21 gauge and 23 gauge needles.

All preparations used the same initial protocol as described above. Some modifications to the subsequent steps were made as conditions were optimised. These variations are indicated in the pertinent "results" sections.

Purification

The cell suspension produced from the trituration step also contained cellular and myelin debris, together with a few small undissociated tissue particles. This was then filtered through a Nalgene filter containing a 40μm steel mesh backing a 20μm nylon mesh, and flushed with 10% FCS in DMEM.

Myelin debris was then removed by centrifugation at 2200rpm for 20minutes through a 9% Optiprep solution in DMEM. The cellular pellet and bottom 1ml of supernatant were resuspended with a 1ml Gilson. This was then washed through the final culture medium once and resuspended in this medium. The early NG2 identification experiments used 10% FCS in DMEM. Later experiments, including all quantitative work, were performed using NSA™ (Euroclone - a high glucose DMEM/F12 based medium) with 2mM L-glutamine, N2 supplements, FGF (10ng/ml), $PDGF_{AA}$ (20ng/ml) and NT-3 (2ng/ml) further supplemented with insulin (final concentration 25μg/ml). Cells were plated onto poly-D-lysine (PDL) coated coverslips and incubated at 37°C in 7% CO_2.

Magnetic Selection and Mitogen Expansion

The protocols used for magnetic selection varied as improvements were incorporated, but were based around pre-existing protocols (kindly provided by Dr J Crang, University of Cambridge) and product data sheets. Initially, the cell suspension obtained following trituration was washed by centrifugation through 10% FCS and then through an Optiprep™ density gradient to remove myelin debris. Cells were then incubated for 30 minutes in filtered A2B5 antibody solution (1:3) at 4°C on a rotating mixer. The cells were then washed and incubated with prewashed anti-mouse IgM coated magnetic beads for a further 30 minutes at 4°C. A2B5 positive cells were removed by repeated selection using a Dynal™ magnet and the beads then digested off the cells with a brief incubation in trypsin, followed by neutralisation with 10% FCS and centrifugation over concentrated Optiprep™ to remove the high density magnetic beads. The cells were then plated onto 4 polylysine coated coverslips, one of which

was also coated with vitronectin, and incubated in NSA-N2 with FGF, PDGF and 1% FBS. (Results Figure 4-16 & Figure 4-17)

The second preparation reintroduced the filtration step through the 20μm filter immediately after trituration, but was otherwise identical. However following magnetic separation the beads were left *in situ* and all cells were grown on poly-lysine coated coverslips in NSA-N2 with FGF2 and $PDGF_{AA}$ without FBS. Wells were flooded with fresh medium 12 hours later, and fresh growth factors were added three times a week by replacing 1/10th of their volume with a 10x solution of growth factors. Every week, old medium was removed sparing a 0.25ml residual and fresh medium replaced. This incubation was continued for 18 days prior to staining. (Results Figure 4-18 to Figure 4-21)

The final protocol was modified in accordance with a published protocol (Windrem et al. 2002). Following both the filtration step and density centrifugation to remove myelin debris, freshly dissociated cells were incubated for 48 hours in an uncoated flask. The medium used was NSA-N2 with FGF2 and PDGF as previously, but with the addition of NT-3. Loosely adherent and non-adherent cells were then washed off with Hanks' balanced salt solution without calcium and magnesium and these cells used in the subsequent separation step. Magnetic separation was performed as above and A2B5+ve cells placed on vitronectin and polylysine coated coverslips and fresh medium added. Growth factors were replaced as described above.

Fluorescence Immunocytochemistry

Adherent cells growing on poly-lysine coated coverslips were removed from their wells and placed on a humidified platform. Surface antigens were stained on living cells by incubation at 37°C for 20 minutes with antibodies diluted in medium containing either 10% serum or 1% bovine serum albumin (A2B5 1:2 dilution, mNG2 1:50, pNG2 1:100) before washing and fixing with 4% PFA. Internal antigens were exposed by permeabilising with absolute methanol at –20°C and then blocked with 5% normal goat serum in phosphate buffered saline at 37°C for 30 minutes. Intracellular antigens were stained overnight at 4°C in phosphate buffered saline (PBS) containing 5% normal goat serum (NGS). Fluorescein isothiocyanate (FITC), tetramethyl

rhodamine isothiocyanate (TRITC), or 7-amino-4-methyl-coumaric acid (AMCA) conjugated class and species specific secondary antibodies diluted in 5% NGS were applied for 1 hour at room temperature. Various controls included the omission of individual primary antibodies (of particular pertinence to multiple staining) and the use of irrelevant antibodies of the same isotype and derived from the same source (eg anti BrDU antibodies in the absence of BrDU incubation). Internal controls were provided by other cellular phenotypes within the mixed cultures. After the last washing step, coverslips were mounted in Vectashield™ (Vector Labs) and sealed with clear nail varnish. Slides were then viewed under using an Olympus IX70 inverted fluorescence microscope with appropriate filters, and photographed using a Polaroid™ digital camera.

Results

Systematic analysis of human mixed glial cultures requires triple staining of multiple parallel coverslips to identify all phenotypes. Because of the critical limitations of tissue volumes from surgical specimens (typically less than $0.5cm^3$) insufficient coverslips were available to stain more than one coverslip with each combination of markers per sample, thus sacrificing statistical power. In addition these protocols were very vulnerable to the failure of a single stain, as the experimental design required simultaneous identification of all major cellular groups to test these hypotheses.
The following results incorporate observations from such experiments where qualitative findings were manifest but where quantification was impossible. In addition, very commonly during the course of an experiment very specifically designed to answer one question, additional observations pertaining to other protperties emerged, but the design of the experiment was such that the secondary observations could not be quantified. Often the same unsought finding was suggested from several different primary experiments. However, attempts to confirm or refute such unquantified observations were frustrated by the limited and sporadic nature of the tissue supply.
In the following, where quantified, figures refer to a *single* comprehensive study of tissue from one patient and, unless stated otherwise, refer to one coverslip for each separate analysis and are thus invariably provisional.

The Identification and Immunophenotype of the Adult Human Oligodendrocyte Progenitor

Sensitivity and Specificity of NG2 as a Marker of A2B5+ Oligodendrocyte Progenitors

A2B5 positive cells could be readily identified in these cultures in line with previous observations (Figure 4-1) (Scolding et al. 1995). These were not rare (e.g. at 8 days 10.4% (±1%) of total cells in these cultures stained for A2B5) and these cells had both rounded and extended morphologies, the latter predominantly uni- or bipolar. The rounded cell population would be expected to include processed cells that had lost processes during dissociation. With time in culture there was a progressive loss of the A2B5+GFAP- progenitor phenotype and a corresponding rise in A2B5+GFAP+ "type

2" astrocytes (Figure 4-2). This was quantitatively demonstrated between 5 and 8 DIV (Figure 4-3 & Figure 4-4). One explanation for this was that a significant proportion of progenitors might be differentiating into "type 2" astrocytes even in serum free conditions.

NG2 is trypsin sensitive (Nishiyama et al. 1991), and no NG2 staining was seen within the first 24-48 hours after enzymatic dissociation. Furthermore cellular identification became easier with time *in vitro,* as cellular adhesion, morphology and staining clarity improved. Thus time-points of 5 days *in vitro* (DIV) and 8 DIV were used for quantitative analysis.

NG2 staining was evident in 6.9% (±0.8%) of total cell numbers. However almost all NG2+ cells had an extended morphology and they made up a correspondingly greater proportion of the non-rounded cell population.

When the A2B5 +ve GFAP -ve progenitor population was examined, only 31.3% (±8.2%) of cells also stained with NG2 (the *sensitivity* of NG2 as a marker of oligodendrocyte progenitor cells).

Of the population of NG2 +ve cells present at 5 DIV, 38.2% (±4.8%) were A2B5 +ve. However half of these A2B5+NG2+ cells were co-positive for GFAP – ie only 17.6% (± 3.8%) of the total NG2 +ve cells had the traditional immunophenotype of OPCs (A2B5+ GFAP-) (Figure 4-3). By 8 DIV this figure had fallen to 9.8% (±2.9%) (the *specificity* of NG2 as a marker of OPCs) while the proportion of the total NG2+ population staining for A2B5 remained broadly the same, due to a corresponding rise in the number of NG2+ "type 2" astrocytes (Figure 4-4).

PDGFαR expression in primary glial cultures

The low figures for both sensitivity and specificity prompted further inquiry. Another antigen that has been used as a marker for identifying oligodendrocyte progenitors in tissue sections is the PDGF α-receptor (PDGFαR) (Hart et al. 1989;Pringle et al. 1992). Rodent studies have suggested that this antigen expressed in cells at both the pre-progenitor and progenitor stage (Ben Hur et al. 1998) and thus overlaps with A2B5, but is expressed and lost first. This stage of oligodendrocyte development is strongly influenced by exogenous PDGF-AA, which is a potent mitogen (reviewed in (Casaccia-Bonnefil & Liu 2003)). There are some reports that it may be expressed by certain neurons (Vignais et al. 1995), but as discussed earlier, this is not directly relevant to primary glial cultures from adult brain as neurons do not survive the

dissociation protocol. The use of PDGFαR as a marker of the human OPC *in vitro* has yet to be established. However, if NG2 antigen was expressed earlier than A2B5 then it might be expected to correlate better with PDGFαR, which has an earlier expression profile in the oligodendrocyte lineage (Ben Hur et al. 1998). Interestingly, there is also evidence that PDGFαR co-localises with NG2 and may have some role in PDGF signalling (Nishiyama et al. 1996a;Nishiyama et al. 1996b).

It was decided to investigate the possibility that NG2 and PDGFαR were labelling the same population of cells. When counted independently, PDGFαR was expressed by 29% (±1.9%) of the total cell population, most of which were rounded adherent cells. This compared with 10% (±1.0%) of cells staining with A2B5 and 6.9% (±0.8%) with NG2 in parallel slides. The latter marker stained cells with an almost exclusively extended morphology. Differentiation between the traditional OPC phenotype and the "type 2" astrocyte was not possible in this analysis.

When the staining profiles of all three were compared on cells with an extended morphology (excluding cells that were unequivocally microglia on morphological grounds) a complex overlap between the populations was observed (Figure 4-6). This did not support the hypothesis that NG2 was labelling an identical population to that of PDGFαR. Notwithstanding the reports suggesting a particular association between NG2 and PDGFαR, one might expect some variation in the timing of different antigen expression as cells progressed through the lineage. The data presented to this point could be reconciled with the original hypothesis that NG2 staining, along with A2B5 and PDGFαR, are specific markers of the early oligodendrocyte lineage *in vitro*, if their expression is broadly sequential, but with a degree of variation between individual cells. The previous Venn diagram has been modified to illustrate this idea in cartoon form, using normal curves to demonstrate variable expression (Figure 4-7).

NG2 staining of the oligodendrocyte lineage – comparison with morphology

The oligodendrocyte lineage in rodents is traditionally renowned for the reproducible and strictly ordered sequence of lineage markers that appear, and in some cases disappear during maturation. This is mirrored by increasing morphological complexity; pre-progenitors are often unipolar, progenitors typically bipolar and later precursors develop increasing numbers of simple and then progressively ramified processes (Figure 4-8). We decided to use this to investigate the possibility that NG2

was marking a stage of oligodendrocyte development either before or beyond that of A2B5.

We hypothesised that an expression earlier or later in the lineage would be manifest by an NG2 expression pattern that was skewed in relation to morphological complexity. This could only be directly addressed in relation to number of processes manifested by the sub-population of cells that had identifiable (and hence countable) processes. For this purpose processes were defined as narrow extensions clearly demarcated from the soma which extended for > 1 nuclear diameter beyond the point of demarcation. Processes were classed as branched (and hence counted as one) if the division was located distal to the demarcation point, but counted separately if the division was proximal.

The number of processes of each NG2+ oligodendrocyte progenitor, NG2-ve oligodendrocyte progenitor and the A2B5-ve NG2+ cell was recorded. As previously, the oligodendrocyte progenitors were defined by the expression of A2B5 and the absence of GFAP staining: When the morphology of these populations were compared, we did not see a more primitive phenotype (fewer processes) in any of the populations to support this hypothesis. Clearly this result needs repetition before this conclusion can be claimed. (Figure 4-9).

Those cells that could not be included in the above analysis nevertheless gave important clues as to the expression patterns of NG2. Two particular morphologies of NG2 positive, A2B5 negative cells were highlighted by this. There was a preponderance of this antigenic phenotype in those cells classified as growing in clumps. These cells were assigned a discrete class irrespective of process number both because it was felt they might have a unique origin and because morphological definition was prevented by the proximity of neighbouring cells. Stem cells and early progenitors appear to grow as aggregates in tissue culture, but may later adhere and disperse on permissive substrates (Ben Hur et al. 1998). Clumped cells may therefore represent such cells that have recently started to adhere. Although this preponderance would not support the expression of NG2 later in the lineage than A2B5, it might be explained by an earlier pattern of NG2 expression.

The second group of NG2+A2B5- cells comprised a readily distinguishable population of flattened cells with elongated, curved (reverse scalloped) or clawed morphology (Figure 4-12). These cells could not be classified as processed as defined earlier because the flattened extensions of the soma were noticeably broader and were not

clearly demarcated from the soma (occasional cells could, and were classified as processed, while still possessing some of these characteristic features - Figure 4-12). With occasional exceptions, this morphology was unlike that of previous descriptions of oligodendrocyte lineage cells growing *in vitro* and did not readily support the hypothesis that NG2 was a selective marker of this lineage.

NG2 staining of the oligodendrocyte lineage by immunophenotype

An alternative approach to address the possibility that NG2 labels oligodendrocyte lineage cells, but at a slightly different stage to A2B5, is to look at earlier and later antigenic markers. In rodents, neural stem cells and oligodendrocyte pre-progenitors label with the intermediate filament protein nestin but this is lost at or around the progenitor stage after the acquisition of A2B5 (Ben Hur et al. 1998). Nestin is not considered specific to the oligodendrocyte lineage; indeed many regard its expression as an indicator of pluripotentiality (Reynolds et al. 1992). A homologue of rodent Nestin is expressed in human cells and reports of the staining characteristics of stem like cells from human source tissue suggests that it too is expressed by immature cells (Johansson et al. 1999), although may be lost at an earlier stage in humans (Scolding et al. 1995). The O4 antibody on the other hand is regarded as a specific oligodendrocyte lineage marker both in rodents and humans. It predominantly labels sulfatide, an antigen first expressed during the later progenitor stage and one that continues to be expressed right through to the mature myelinating oligodendrocyte (Zhang et al. 2000). Thus the combination of these markers might, according to traditional dogma, be expected to label all potential and actual oligodendrocyte lineage cells. If the proposed hypothesis is correct, all NG2+ cells would be expected to co-express one of the other three antigens (Nestin, A2B5 or O4) (Figure 4-8).

This was investigated in a series of triple labelling experiments. Nestin positive cells were numerous compared with other phenotypes, and made up 15.5% of total cell numbers (Figure 4-10). The majority of these cells were rounded and had little evidence of protoplasmic extensions or processes. There was an identifiable overlap between nestin and A2B5; consistent with previous observations in rodents that nestin is lost after A2B5 is acquired (Ben Hur et al. 1998). Furthermore there was a proportion of cells that labelled for NG2 and nestin that did not label for A2B5, suggesting that NG2 may be expressed earlier in the lineage than A2B5 (Figure 4-11).

However, this proportion made up only 8.3% of the total NG2 staining population – leaving a large proportion of NG2 positive cells without positive identification.
O4 stained 12% (±3%) of adherent cells, and a similar proportion of total cell numbers (11%). A small proportion of these cells co-labelled for NG2 but this made up only some 5% of total NG2+ numbers. Again, no O4 staining was seen on the morphologically distinct, NG2+ clawed cells.
These studies suggest that NG2 may start being expressed earlier than A2B5 in the oligodendrocyte lineage but may, like A2B5, extend into the period of O4 expression. However it fails to explain all the observed discrepancies between NG2 and putative human oligodendrocyte markers, and as such, conclusions about relative timing should be interpreted with caution.
Taken together, these findings suggest that NG2 expression may not be restricted to the oligodendrocyte lineage, as defined by established (rodent) oligodendrocyte markers. We attempted to identify other cell populations that might be expressing NG2.

<u>The identity of A2B5- NG2+ cells in primary glial cultures</u>

The value of NG2 as a specific marker of oligodendrocyte progenitors is not universally supported. Previous reports have identified NG2 staining on a number of other cell populations that would be expected to contribute to a mixed glial culture. The most consistent report is of co-staining of NG2 with endothelial cells (Pouly et al. 2001). Some other observers have suggested that microglia also express this antigen – although this study rested heavily on FACS analysis, and the images published of immunostained cells showed rounded cells with little or no detail of antigen distribution or morphology. These findings have been refuted – at least as far as they pertain to microglia *in vivo* (Chang et al. 2000a;Nishiyama et al. 1999) by those who maintain that NG2 is a useful oligodendrocyte progenitor marker. The observation of NG2 expression only on a subgroup of activated macrophages may go some way towards resolving this discrepancy (Bu et al. 2001).
To test the hypothesis that NG2 is a specific marker of oligodendrocyte progenitors *in vitro*, it was necessary to use multi-labelling techniques. Only three antigens at one time could be identified with any security, so careful analysis of identical, parallel samples of mixed glia from the same preparation was necessary.

NG2 stains type 2 astrocytes, fibroblasts and endothelial cells

As previously mentioned, NG2 stains a proportion of type 2 astrocytes in cell culture in common with A2B5 (Figure 4-2). The role of these cells *in vivo* has been much debated. However it seems increasingly likely that oligodendrocyte progenitors develop exclusively into oligodendrocytes in physiological situations (Groves et al. 1993), although type 2 astrocytes may occur in some pathological situations (Franklin & Blakemore 1997). We found that 50% (±0.9%) of type 2 astrocytes seen *in vitro* labelled with NG2, a proportion that accounted for 11% (±2%) of total NG2+ cell numbers at 8 DIV (Figure 4-4). Very occasionally, cells with the immunophenotype of type 1 astrocytes labelled with NG2, but these cells did not have typical type 1 astrocyte morphology. Most type 1 astrocytes were negative, and the question of NG2 labelling of these cells requires further study.

Fibroblasts occur variably in human glial cultures, and are believed to originate from larger blood vessels or contaminating meningeal fragments. Although usually present in small numbers, they proliferate rapidly and can quickly overrun the cultures(Figure 4-13). They are usually identifiable by their distinct flattened morphology, and can be stained with antibodies directed against Thy 1, also known as CDw90 (Antel 2003). All of the Thy-1 positive cells in these cultures also stained for NG2 (control experiments excluded cross-reactivity between the secondary antibodies) (Figure 4-13). These cells looked very different from the population of clawed or elongated NG2+ cells that were described in the last sections, and remain to be positively identified. In total Thy1+ cells made up 9.1% (±4.3%) of the total NG2+ population at 8 DIV.

Endothelial cells, originating from cerebral vasculature grew in small clumps in these mixed glial cultures. They were identified by strikingly granular immunofluorescence when labelled with antibodies against intracellular von Willebrand's Factor. These cells also co-stained for NG2, as previously described (Pouly et al. 2001). However at 8 DIV only 7.9% of the total NG2+ population were identified as endothelial cells.

Neurons are not readily grown in primary glial cultures from adult tissue without specific growth conditions designed to expand stem cells – and indeed no neuronal markers were expressed. However, neurons did appear to arise *de novo* from aggregate cultures as described later. These are believed to represent the products of stem cell proliferation and differentiation. Several co-staining experiments were performed on this culture system (see stem cell chapter), but no co-expression of NG2 was observed

on any cell labelling with β_{III}tubulin, neurofilament or microtubule associated protein 2 (MAP-2).

NG2 antibodies bind to a small population of microglia – but the staining pattern suggests that this is predominantly artefactual

Microglia are often identifiable in culture on morphological grounds alone. They are usually elongated/bipolar or amoeboid, but have a prominently granular cytoplasm which autofluoresces (Antel 2003). They can also be stained with antibodies directed at a number of different surface antigens. These include the common leukocyte antigen CD45, CD11b (Akiyama & McGeer 1990) and CD11c (Antel 2003;Pouly et al. 1999). The latter is present only on activated microglia – but the process of dissociation is sufficient to induce this state in previously resting cells. However, live staining (required for both CD11b & CD11c, both of which are formaldehyde sensitive (product data sheet)) can be associated with pinocytosis of antibodies – leading to an artefactual punctate staining pattern. This can be reduced by incubation at 4°C for 30 minutes, but small amounts are still sometimes present unless exquisite care is taken in cooling both coverslips and antibody solution prior to and during incubation.
We found this type of punctate, artefactual NG2 staining commonly occurred on microglia with less than rigorous adherence to this protocol. However, even when this was excluded there was evidence of very weak surface staining, albeit easily distinguishable from standard NG2 staining, on a small proportion of cells (Figure 4-14). Using double labelling, and the most inclusive criteria for determination of positive NG2 staining, microglia made up only 4.9% of the total NG2+ population. The clawed or elongated population of NG2 positive cells indicated earlier have a bland homogeneous cytoplasm quite unlike those of microglia and never stained for microglial markers.

Does NG2 stain a novel population of glial cells?

When the figures discussed above are combined, there is a significant proportion (27%) of NG2+ cells that remain unaccounted for (Figure 4-15). Simultaneous triple staining of identical, parallel coverslips enables multiple separate populations in a mixed culture to be individually assessed, but makes rigorous assessment of an unstained population difficult. However, there is much to support the assertion that a distinct population of NG2+ cells falls outside the other known phenotypes. Firstly, while unstained, these cells are identifiable on all slides by means of a characteristic

morphology. They are flattened, with two or more elongated, process-like extensions to their cell bodies. These extensions are unlike the processes of other cells – they are wide and flattened, and often have one or more border with a high contrast, arcuate edge, which suggests bowing of the membrane between two points of attachment. In fact these cells can be easily identified on phase contrast alone, and their immunoreactivity to NG2 antibodies (and unreactivity to other antibodies) predicted and confirmed under fluorescent microscopy. These observations predate the systematic assessment by several months, and were confirmed (but not quantified) several times with all antigens tested.

Although the expression of NG2 by several other phenotypes in glial cultures undermines the use of this antibody to identify OPC's *in vitro,* only the weak staining of microglia and the staining of a tiny subpopulation of type 1 astrocytes would be expected to contribute to the population of cells identified by NG2 *in vivo*. Endothelial cells and fibroblasts are either clearly identifiable in the vessels themselves, or absent from the brain parenchyma, so are unlikely to be confused. In addition, the authors of the report detailing unexpectedly large numbers of NG2+ putative oligodendrocyte progenitors (Chang et al. 2000a) assert that they had excluded microglia as a source of the NG2 staining. However the contribution made by the, as yet unidentified, clawed or elongated cells described above, is of some significance. The authors assert that these are oligodendrocyte progenitors. Our findings using a number of oligodendrocyte or oligodendrocyte progenitor specific markers do not allow this to be substantiated. However, if confirmed, this would suggest a much larger population of these cells than had previously been identified. There is a pressing need to characterise these cells further.

Magnetic bead separation of A2B5 positive cells yields a population of bipolar NG2-positive, A2B5-negative cells which proliferate.

Having concluded from these experiments that there is a population of NG2+ cells in mixed glial cultures that is not easily defined using traditional immunocytological methods, it was decided to pursue the assertion that these are cells of the oligodendrocyte lineage using alternative techniques. One possible explanation for the difficulties encountered in positively identifying these cells is that the traditional antigens used for identification are being lost in culture. This hypothesis is difficult to test directly with multiple staining at a single time point (as used above). As most cells

were being stained after 5 or 8 days in culture (to overcome the problem of NG2 loss during enzymatic dissociation) there could be a change in antigen expression before staining was performed. One such change has already been reported – the acquisition of GFAP expression by A2B5 cells. It would be equally plausible for cells, initially positive for A2B5, to lose surface antigen expression and thus stain negative for this antibody. It was decided to pursue this hypothesis further while simultaneously addressing the second major hurdle outlined in the introduction – that of isolation and purification of oligodendrocyte progenitors. Cells were therefore isolated on the basis of A2B5 expression using immuno-magnetic cell selection.

A series of methodologies was used as successive technical problems were addressed. The first protocol sorted cells directly after dissociation as originally described (Wright et al. 1997). This maximised the cell count prior to selection, but involved selecting cells that may have had some of the A2B5 antigens stripped off by the trypsin. Staining CG4 cells immediately after passaging with trypsin suggested that the antigen was significantly down-regulated, but returned over 12-24 hours. Paucity of A2B5 staining immediately after dissociation of human cells (data not shown) suggested that a similar process was occurring in these cultures. This was surprising in that the original reports suggested that A2B5 antigens are resistant to trypsin (Eisenbarth et al. 1979). In the first protocol, the 20μm filter step used for other work was omitted because of concerns about cell loss in the apparatus. However this might have allowed small, undissociated clumps to pass through into the final suspension. The yield was good and the selected cells grew well in the medium, which also contained a low concentration of serum (1% FBS) to improve viability. Furthermore the cells proliferate under the influence of FGF2 and PDGF$_{AA}$. Interestingly, the cells adhere better to themselves than to the poly-lysine substrate, so forming small loosely attached aggregates.

At this point two populations could be discriminated on the basis of morphology and immunophenotype. One population was made up of large cells with thick long processes that formed a complex network. These stained strongly for A2B5 and GFAP, and fulfilled traditional morphological descriptions of type 2 astrocytes (Raff et al. 1983;Scolding et al. 1995). The second population was made up of small, predominantly bipolar cells that dispersed across the surface. Notably these latter cells made up the major proportion of the aggregate population. These cells were uniformly

NG2 positive, but, somewhat surprisingly, considering the selection process, they were predominantly A2B5 negative (Figure 4-16 & Figure 4-17).

The success of this experiment was encouraging, but the result needed confirmation, and two potential loopholes were apparent. The first related to the medium - these cells were growing in an environment containing serum, albeit at a low concentration. It was decided to try to eliminate this and so remove the uncertainties associated with undefined media. In addition, the assertion that aggregates were forming *de novo* in this culture system could be challenged by the absence of adequate initial filtration. The second modification, incorporating these two changes, confirmed that A2B5-selected cells produce two populations when grown under the influence of FGF and PDGF, even in the absence of serum. These cells showed similar characteristics to those grown in the presence of serum, although the yield was substantially smaller. Again small aggregates were formed and there was evidence of proliferation *in vitro* from serial observation. The cells forming the aggregates here were convincingly NG2 positive (Figure 4-18 & Figure 4-19) and the dispersed cells assumed a variety of morphologies.

Of considerable interest was the morphology of the NG2 cells produced in this way. Many of these cells were elongated with broad processes, others were flattened with multiple clawed, process like extensions. Most of these cells had the characteristic arcuate, phase-bright edges that resembled the unidentified NG2+ cells from the previous experiments. Furthermore, these cells grew in colonies containing both elongated and clawed cells, which suggested that they might be variations of a common phenotype, rather than distinct cells.

This combination of morphology, immunophenotype and presumptive previous immunopositivity for A2B5 suggests that cells of the population in question had either lost A2B5 staining, or were progeny of the A2B5 positive population. Both interpretations indicate that they belong to the oligodendrocyte lineage. Furthermore their growth in colonies suggested that these cells might be proliferating in these conditions, a provocative observation if it could be proven.

Cells purified on the basis of A2B5 expression incorporate BrDU with appropriate mitogens

During this work, several variants of the basic media, growth factors, and substratum were tried in parallel with the above experiments. The attempts to expand primary,

unsorted human oligodendrocyte progenitors in defined media with other cells of a mixed glial preparation were broadly unfruitful. There were occasional clusters of 3-4 A2B5+GFAP- cells in the mixed cell cultures, but little else to suggest *in vitro* proliferation. However oligodendrocyte progenitors are inherently motile, so the possibility of rapid dispersal of newly formed progenitors was considered. Attempts were made to label with BrDU and identify dividing cells, but no convincing evidence of cell division was observed.

However, when the A2B5 positive cell population was magnetically selected out and grown at high density, these cells did show signs of proliferation and formed aggregates. However this preliminary work had been performed without the filtration step and in medium containing small amounts of serum (see above). Furthermore, unlike the other cultures which were from temporal lobe samples, these cells were derived from cerebellar tissue and this work therefore carried certain caveats as outlined above. In addition, these cells were selected immediately following enzymatic dissociation when antigen expression would be expected to be low, and to combat this, selected cells from the entire sample of brain tissue (~0.6 ml yielding approximately $3.6x10^6$ cells prior to selection) were concentrated onto 4 coverslips.

Despite the presence of poly-lysine to promote adherence, these cells were observed to form partially adherent aggregates with an interconnecting network of elongated processes. Subsequent staining demonstrated a mixture of A2B5+GFAP+ cells and a large population of bipolar NG2+ cells that did not appear to stain for A2B5. However, the latter appeared to be the major components of the aggregates, and when dispersed often formed colonies of similar cells, suggesting recent division.

Serum is known to contain a number of different growth factors, some of which remain undefined. Thus any demonstrable proliferation cannot be safely attributed to the recombinant growth factors alone. These experiments were confirmed with a second sample using both a filtration step, to ensure all aggregates were newly formed, and medium free of all traces of serum. Again significant proliferation was seen, and similar populations of NG2+A2B5- cells were identified growing in colonies. Unfortunately, attempts to stain for BrDU and NG2 were unsuccessful, so cell proliferation could be inferred but not proven.

Notwithstanding the caveats expressed in the former paragraph, the growth of this population of purified cells into aggregates (and adherent colonies) was indicative of significant proliferation under these conditions. However cellular yield remained low

making analysis problematic, and a final modification to the protocol was introduced to address this. A2B5 antigens are known to be lost during enzymatic dissociation – so a higher yield might be expected if the selection process was delayed to allow the reacquisition of antigens, as proposed by Windrem et al (Windrem et al. 2002). The details of this latter protocol also vary slightly from that used in earlier attempts (see methods section).

Rather surprisingly this yielded substantially fewer cells than before. This could not be easily attributed to poor survival during the initial dissociation, as the cell yield after dissociation was roughly comparable. Furthermore those cells that survived had a rather different morphology to the cells grown after immediate dissociation. These cells were much closer to the traditional description of oligodendrocyte progenitors growing *in vitro* – they had mostly one to three narrow processes (often quite long) and a rounded soma. Interestingly, the presence of magnetic beads, while obscuring the view under phase contrast, did suggest considerable motility of some of these cells – many of which had cleared a bead-free halo off their neighbourhood. In addition some pairs of cells became evident, which was suggestive of cell division.

To confirm proliferation the cells were incubated with bromodeoxyuridine (BrDU) for 24hours. Technical difficulties with the use of certain surface stains and BrDU prevented adequate demonstration of NG2 co-staining with BrDU, but PDGFαR staining was reliable in this situation. PDGFαR+ cells could be demonstrated to incorporate BrDU with this technique (Figure 4-21).

Discussion

The presence of oligodendrocyte progenitors in the adult human brain has been well demonstrated (Roy et al. 1999;Scolding et al. 1995;Windrem et al. 2002), but the range of different putative markers to identify these has lead to discrepancies in these findings. Are these different markers equally valid? Issues of antigen sensitivity and specificity have implications for cellular identity that are of more than just academic interest. Are oligodendrocyte progenitors rare, as originally suggested (Scolding et al. 1998), or one of the more abundant cells of the adult nervous system (Chang et al. 2000b)? The answer to these questions will have important implications for therapeutic remyelination strategies. This work helps to clarify certain points and identifies questions for future studies.

From this work, NG2 appears neither to be a sensitive marker, nor a specific marker for oligodendrocyte progenitors growing *in vitro*. Provisional results suggest it labels under a third of these cells, as defined by traditional markers, while also staining astrocytes (both "type 2" and possibly occasional "type 1"), endothelial cells and fibroblasts. It stains microglia only very weakly, but with less than rigorous methodology, can identify large numbers of these phagocytes. Furthermore, there is evidence of NG2 expression earlier in the oligodendrocyte lineage than A2B5, although this, and the relationship between PDGFαR and NG2, requires further work. In addition, NG2 appears to identify a novel population of cells in these cultures that are also prevalent in a population magnetically selected for A2B5 expression. The identity of these cells warrants further discussion. Finally there is evidence that purified A2B5+ cells do proliferate in serum-free medium under the influence of mitogens predicted by rodent work.

In vitro work has contributed a wealth of data towards our understanding of the biology of neural and glial lineages, but *in vivo* confirmation of these findings and further exploration of progenitor biology *in situ* has been complicated by the difficulty of reliably identifying precursors. NG2 proteoglycan has previously been shown to be a useful marker for oligodendrocyte progenitors in the developing rat *in vitro* (Stallcup & Beasley 1987) and *ex vivo* (Nishiyama et al. 1996a;Ong & Levine 1999). However, the staining characteristics of adult rodent oligodendrocyte progenitors differ from those of neonatal progenitors (Ffrench-Constant & Raff 1986;Wolswijk et al. 1991a;Wolswijk & Noble 1989). In addition, adult human oligodendrocyte progenitors

have been reported as differing from their adult rodent counterparts, particularly in their absence of nestin or vimentin expression (Armstrong et al. 1992;Ffrench-Constant & Raff 1986;Scolding et al. 1995;Wolswijk & Noble 1989) and their failure to divide with mitogens which would cause significant expansion of their rodent counterparts (Scolding et al. 1995). Despite these differences, we show here that the NG2 proteoglycan is expressed by some cultured cells of the adult human oligodendrocyte lineage, including a proportion of both A2B5+ve precursors and A2B5/GFAP co-positive ("type 2") astrocytes.

The finding that some adult human oligodendrocyte progenitors exhibit cell surface expression of the chondroitin sulphate proteoglycan NG2 provides further illustration of the important biological similarities between these cells and their neonatal rodent counterparts (Figure 1-5). They also share a common bipolar morphology, A2B5+ve GFAP-/GalC- immunophenotype, capacity for differentiation *in vitro* into either oligodendrocytes or A2B5/GFAP copositive (or "type 2") astrocytes, and proliferative activity stimulated by syngeneic astrocytes (Scolding et al. 1995). However the significant lack of specificity, and the limitations of sensitivity evident from our observations concerning NG2 challenges the exploitation of NG2 expression as a marker of adult human oligodendrocyte progenitor cells in tissue sections. It should be emphasised that considerable caution must be exercised in this respect.

Initial studies of NG2 showed expression not only by cultured rat oligodendrocyte progenitors but, significantly, also by A2B5/GFAP copositive type 2 astrocytes (Stallcup & Beasley 1987), so that the similar positive staining of human A2B5/GFAP copositive astrocytes by anti-NG2 antibodies is not unexpected. It is therefore apparent that NG2+ve cells in tissue sections of rat or human origin must be clearly shown not to be astrocytes before it can be concluded that they are indeed oligodendrocyte progenitors. This is particularly true when the injured CNS is studied, since it is in response to injury that the type 2 astrocyte is proposed to arise (Franklin & Blakemore 1997); other researchers have also demonstrated the presence of A2B5/GFAP co-positive astrocytes (using tissue print preparations) in the human CNS (Scolding et al. 1999).

Generally, studies using anti-NG2 antibodies to identify progenitors have addressed this difficulty using GFAP antibodies to exclude an astrocyte identity of NG2 positive cells (Keirstead et al. 1998). It is important to recall, however, that, with immunocytochemical techniques, the majority of astrocytes in the normal rodent and

the normal human CNS are GFAP-negative (Eddleston & Mucke 1993). Therefore, ultrastructural investigation might helpfully supplement the use of anti-NG2-based immunocytochemistry as a tool for identifying progenitors in studies of normal CNS tissue. Furthermore, since GFAP-reactivity has not been confirmed as an inevitable consequence of astrocyte reactivity (Eddleston & Mucke 1993) - reactive gliosis can occur in the absence of GFAP (Pekny et al. 1995), and GFAP expression can acutely diminish during, for example, viral infection (Kennedy & Fok-Seang 1986) - caution might also be advisable when using NG2 as a progenitor marker in the injured or inflamed CNS.

This work also confirms the previous observations that cerebral endothelial cells express NG2. These too need to be excluded from any analysis of progenitors in tissue slices although their very characteristic location is unlikely to cause confusion for the careful observer.

The current observations on microglia may go some way to explaining the discrepancy between various reports of NG2 expression in this cell type (Chang et al. 2000a;Pouly et al. 1999). The pino/phagocytotic properties of these cells are well known, but it is widely assumed that this can be adequately inhibited by temperature alone. As demonstrated, this may not be as secure as is often believed, causing particular problems when cells are analysed electronically rather than visually. (NB this artefact is caused by viable cells and so will not confound fixed tissue section work). Furthermore the diffuse, low intensity NG2 staining seen on a small proportion of these cells may not be explained on this basis alone. It should be re-emphasised that this staining is of a magnitude unlikely to cause confusion when compared directly with the expression seen on oligodendrocyte progenitors.

An important observation from this work is the presence of significant numbers of NG2 positive cells that have defied traditional immunocytochemical identification. Various hypotheses as to their identity were proposed and have, in part, been tested. Firstly, the population does appear to be identifiable, consistent and real. Fluorescent immunocytochemistry may limit the number of antigens identified on one coverslip, but it has a number of significant advantages over other techniques. Most prominent among these are the ability to scrutinise each cell for structure and morphology, expression of three separate antigens independently and the distribution of these antigens (and hence fluorescence) on the cells. These attributes allow this population

to be identified purely on the basis of morphology and NG2 staining pattern – it seems unlikely therefore that they are a phantom population resulting from cumulative random error during population analysis [Figure 4-15]. (However these considerations must be taken into account before attempting to estimate the size of the novel NG2 population, and the experiment needs repeating). It has been provisionally concluded that these cells are not microglia, endothelial cells, astrocytes or fibroblasts, but rather a novel population expressing none of the markers of the above cells.

Secondly it was hypothesised that they belonged to the oligodendrocyte lineage – but at a different stage in development to the A2B5 positive cells. When a single quantitative analysis of morphology was performed, it suggested that the A2B5+ cells and the NG2+A2B5- cells were morphologically dissimilar and the latter group not easily classified because of their flattened, broad-based processes. Might this difference be a manifestation of the physiological role of NG2? The NG2 molecule is known to be involved with cell motility (Diers-Fenger et al. 2001;Niehaus et al. 1999), playing a central role in the processes of adhesion and release (Stallcup & Dahlin-Huppe 2001). The characteristic morphology may be more than just an unrelated characteristic of NG2+ cells –NG2 expression may itself be influencing the morphology *in vitro*.

However, the central question of identity remains. Is NG2 staining cells at a different stage of the oligodendrocyte lineage? The lack of A2B5, nestin or O4 staining for most of this population does appear to refute this suggestion, but the presumption on which this argument is based is not watertight. All these antigens were originally characterised in the rodent (Raff et al. 1983;Reynolds & Weiss 1992;Warrington et al. 1992), and although they have since been demonstrated in adult human work (Armstrong et al. 1992;Johansson et al. 1999;Scolding et al. 1995), the assumption that human cells have the same highly reproducible and strictly regulated pattern of expression has not been unequivocally demonstrated. There may therefore exist stages between Nestin, A2B5 or O4 where human oligodendrocyte lineage cells express none of these antigens. Having said this, both the demonstration of A2B5+Nestin+ cells described here, and A2B5+GalC+ cells described previously (Scolding et al. 1995) limit potential gaps to a subpopulation of cells, and depend on a degree of variation between cells, which while credible, has not been demonstrated. Alternatively, these antigens may be lost in culture. Cells can lose antigens from their surface when they

become cross-linked or clustered. Might this process occur in these cultures – often containing traces of debris from the original dissociation?

Addressing this question by the use of immuno-magnetic selection immediately following dissociation may go some way to answering this. It appears likely from these experiments that the morphologically distinct NG2+A2B5- cells are abundant in a population that originally expressed A2B5 antigens. If so, several possibilities remain. It seems unlikely that they were a contaminant of the immuno-magnetic sorting process as few other cell types were seen in these cultures. They may express these antigens but at a level too low to be identified by fluorescent immunocytochemistry but one sufficient to be selected by immuno-magnetic methods. As mentioned, few cells were unequivocally A2B5+ by immunocytochemistry immediately after enzymatic treatment, yet magnetic separation was effective at isolating cells at this stage into a population that contained only two types of cell – the "type 2" astrocyte and the NG2+A2B5- cells, so this hypothesis is plausible. The latter cells may have lost A2B5 antigens by endocytosis of aggregated surface molecules, perhaps as a consequence of cross-linkage by free antibody in the magnetic selection process. Alternatively, free A2B5 antibody may be released from the beads in a form not recognised by the anti-mouse IgM- fluorescent marker and so block ganglioside epitopes. These might explain the paucity of A2B5+GFAP- cells in these cultures (observed but not quantified), yet the "type 2" astrocytes retain strong surface staining in the same milieu. If these NG2+A2B5- cells are oligodendrocyte progenitors that have lost their A2B5 staining because of the selection process, do they retain the other properties of oligodendrocyte progenitors? Why do these cells not re-express these antigens while A2B5+GFAP+ cells in mixed culture do after enzymatic loss? If the NG2+/-A2B5+ and NG2+A2B5- populations are otherwise identical, why are the latter cells so flattened when compared to putative progenitors in unselected cultures (Figure 4-19)?

An intriguing possibility is that these two cell types may represent different pathways within the oligodendrocyte lineage (Spassky et al. 2000). There is some evidence that separate populations of putative oligodendrocyte progenitors exist in the developing chick, rodent (Fu et al. 2002) and human (Rakic & Zecevic 2003). It is not clear however whether these are alternative stages within the lineage, or represent an entirely separate differentiation pathway. This might help to square the discrepancies between various reports of the percentage of oligodendrocyte progenitors in adult

human brain depending on the marker used. NG2 may be labelling a separate or additional population of oligodendrocyte lineage cells not identified by other antigens. The *caveat* pertaining to its use as a marker of oligodendrocyte progenitors in tissue sections remains.

None of these questions can be answered without further study. However the observation that this cell type appears to proliferate under these conditions suggests a final, plausible explanation. Antigen loss in a dividing population is a well-recognised *in vitro* phenomenon (Louis et al. 1992). It may become apparent that dividing human progenitors *in vitro* do lose A2B5 reactivity while maintaining NG2 expression, a hypothesis that would have far-reaching consequences if proved. Furthermore, the presence of cell division in these cultures itself has enormous implications. If this expansion can be extended, and there is much to suggest that it could (eg presence of aggregates), then the foremost problem outlined in the introduction would have been solved. Isolation of human progenitors from adult human brain could then be followed by immuno-magnetic selection and *in vitro* expansion as described. Cell numbers from the partial aggregate cultures already approach those required for systematic assessment of cellular biology (proliferation, differentiation & migration) and may in time yield a source sufficient for therapeutic purposes.

Future work

The above studies are ongoing and this work needs confirming by repetition. Antigen expression and cellular morphology are important means with which cells can be identified and followed, but they remain surrogates. A cell is ultimately defined by its physiological role, and for oligodendrocytes this means the demonstration of compact, functional myelin formation and axonal support. However, many experiments are anticipated prior to this. Rodent and human oligodendrocytes express a sequence of myelin proteins during development, and the demonstration of some of these together with the expected morphological changes will support their claimed identity. Co-culture experiments in the presence of human neuronal cell lines could be used for myelination studies, which would likely culminate in transplantation studies.

The hypotheses proposed above to explain the observed behaviour require testing, and the more significant results need repeating. Furthermore, it might prove important to ascertain whether these cells can be obtained from other parts of the brain, and

whether origin affects behaviour *in vitro*. The progeny and differentiation capacity of dividing NG2 cells and traditionally defined A2B5+GFAP- progenitors needs clarifying as does the role of the persistently enigmatic A2B5+GFAP+ "type 2" astrocyte. The results of these experiments are eagerly anticipated.

Illustrations

Figure 4-1 Some bipolar cells growing in culture stained for both A2B5 (red) and NG2 (green) (bar = 10μm)

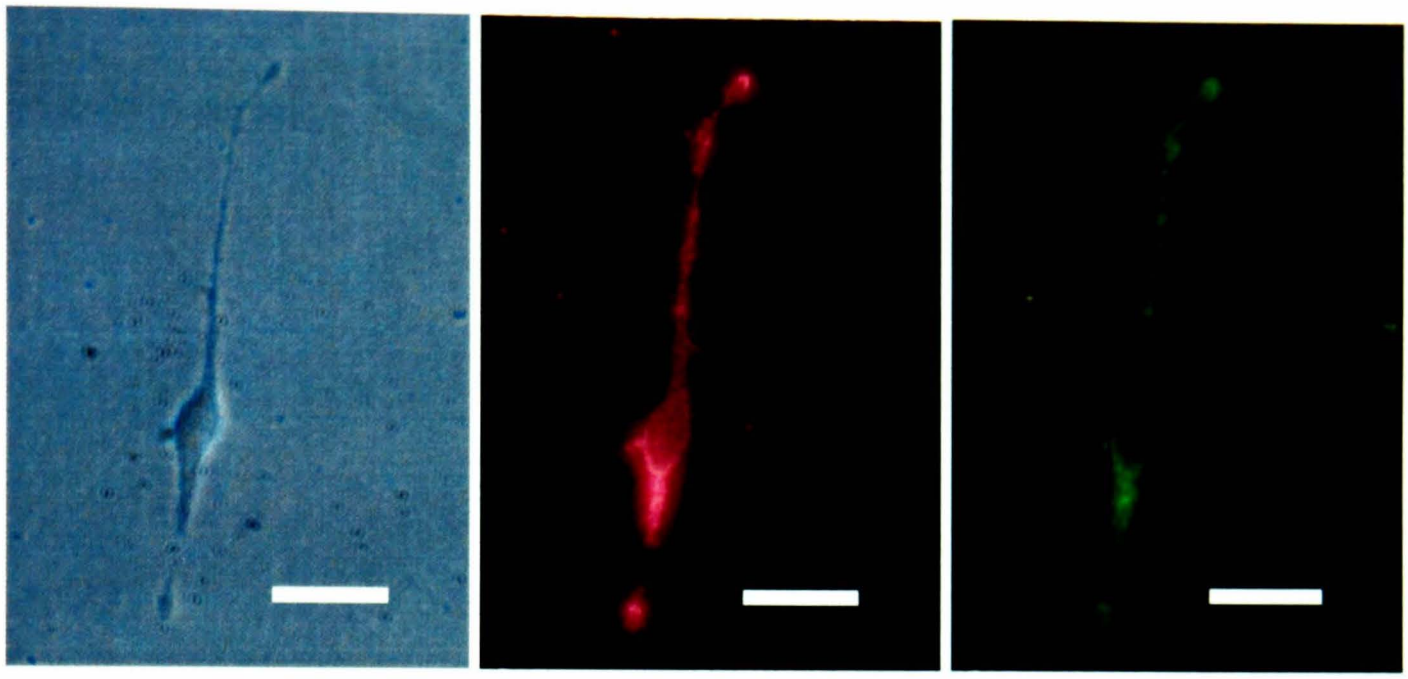


Figure 4-2 Two astrocytic cells can be identified in culture. Type 1 astrocytes (hollow arrow), which have a fibroblast-like morphology and express GFAP (blue), and type 2 astrocytes (solid arrow), which are often large cells with long processes, and express both GFAP and A2B5 antigens (red) (Scale bar = 20μm)

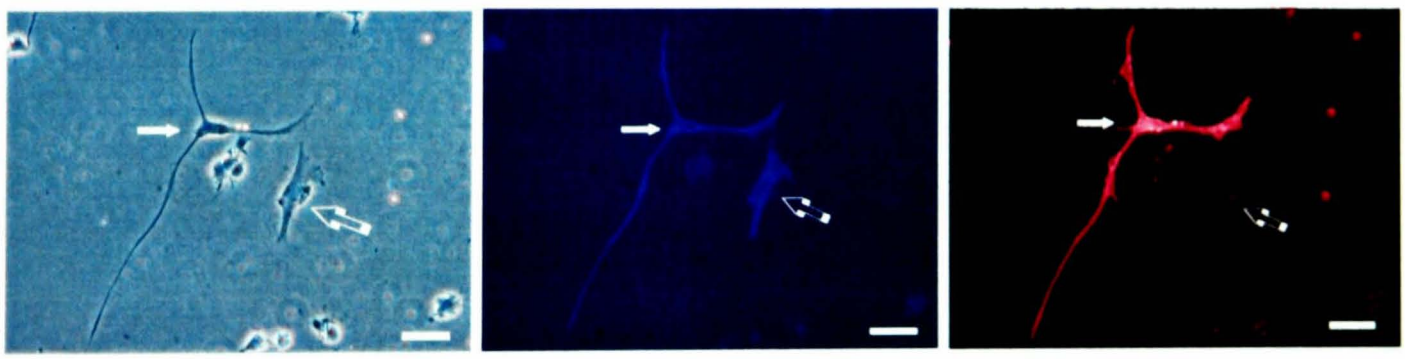


Figure 4-3 Proportion of NG2+ cells staining for OPC/astrocyte antigens of a single representative experiment at 5 DIV. [102 NG2+ cells identified from a population of >1000 cells on one coverslip]

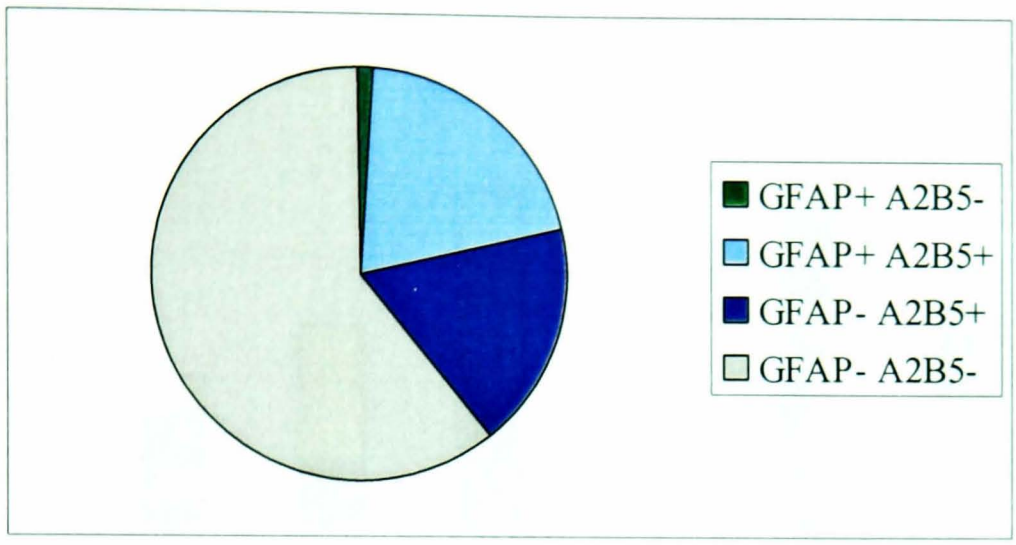


Figure 4-4 Proportion of NG2+ cells staining for OPC/astrocyte antigens of a single representative xperiment at 8 DIV [102 NG2+ cells identified from a population of >1000 cells on one coverslip]

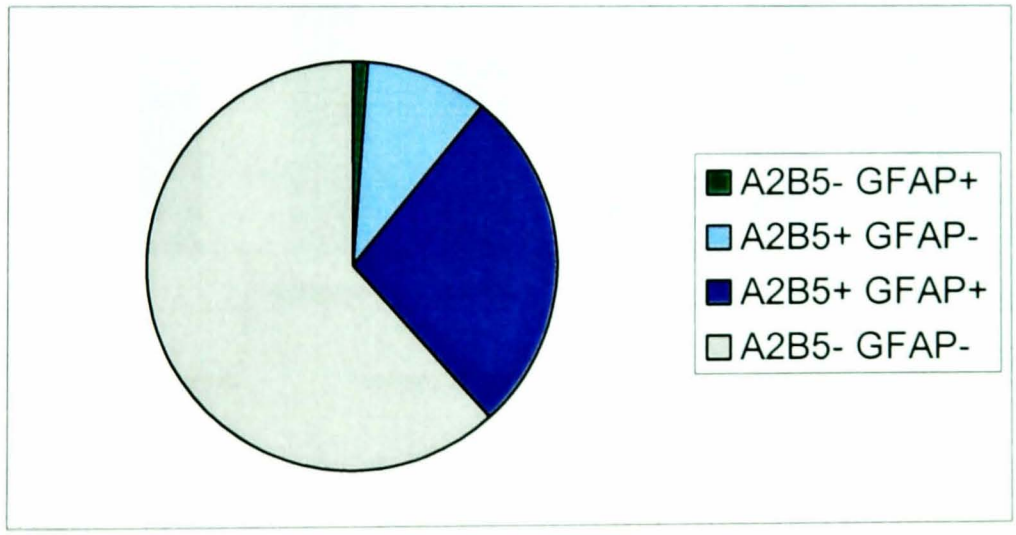
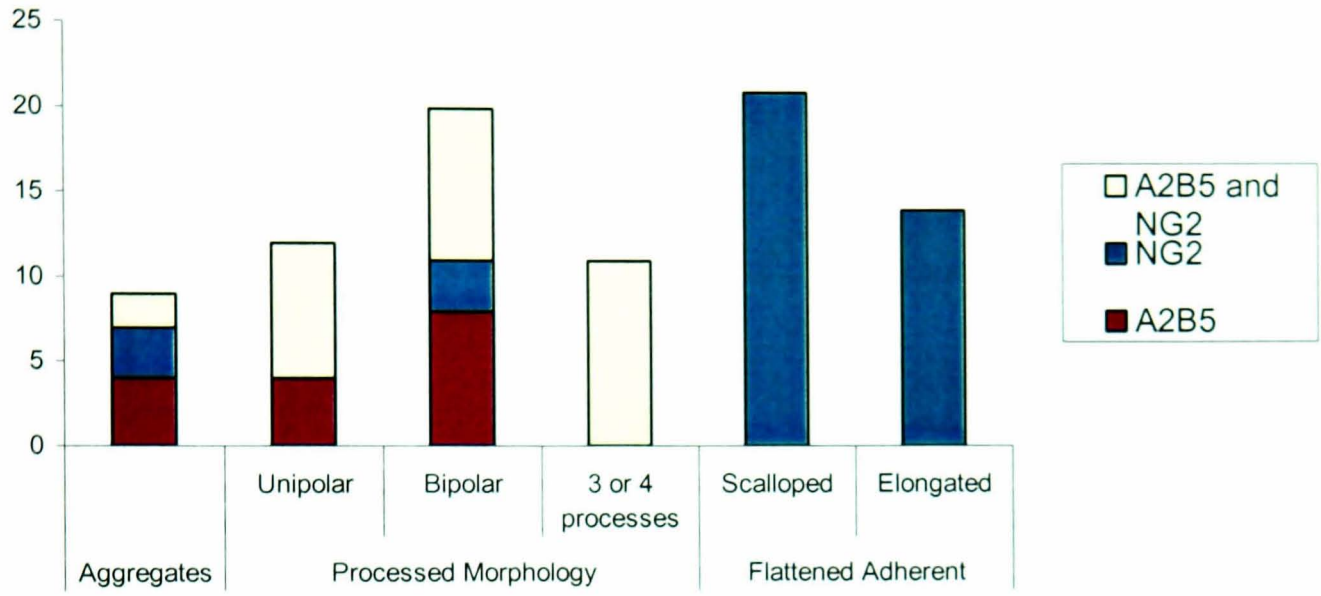
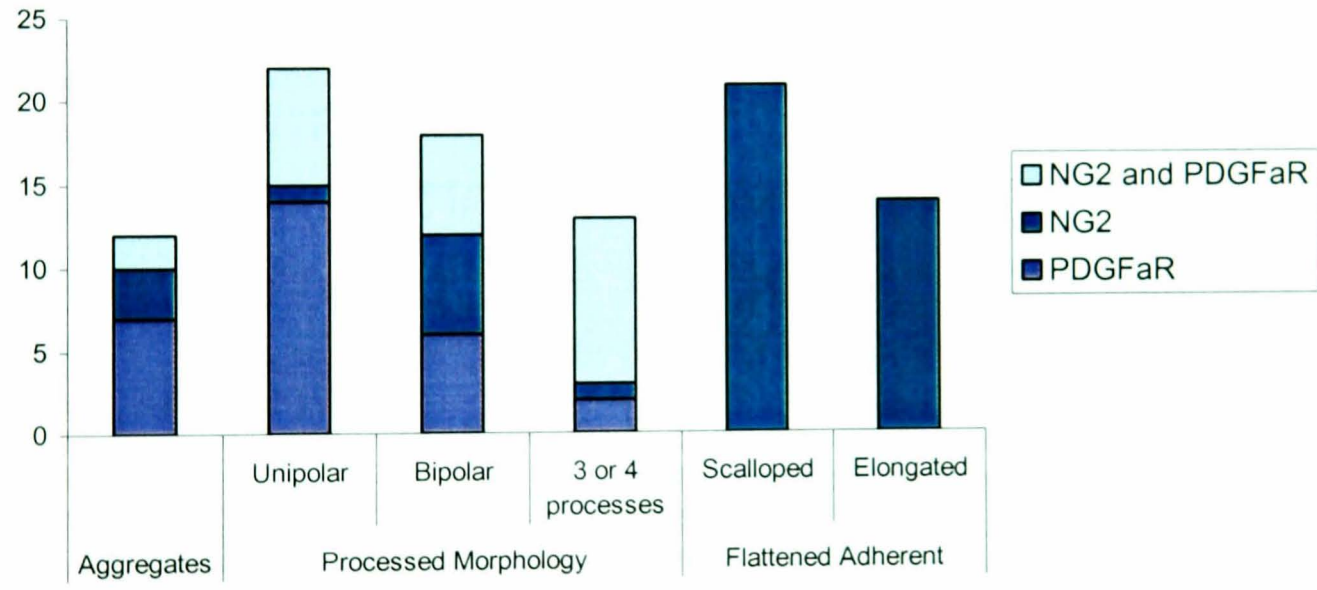


Figure 4-5 Paired comparisons between A2B5, NG2 and PDGFαR plotted against morphology suggest a discreet population of adherent NG2+ cells with elongated or reverse-scalloped/clawed morphology [Comprehensive analysis of all antigenic phenotypes expressed on a single coverslip: 989 cells analysed, immunophenotype and morphology of 106 labelled cells recorded]





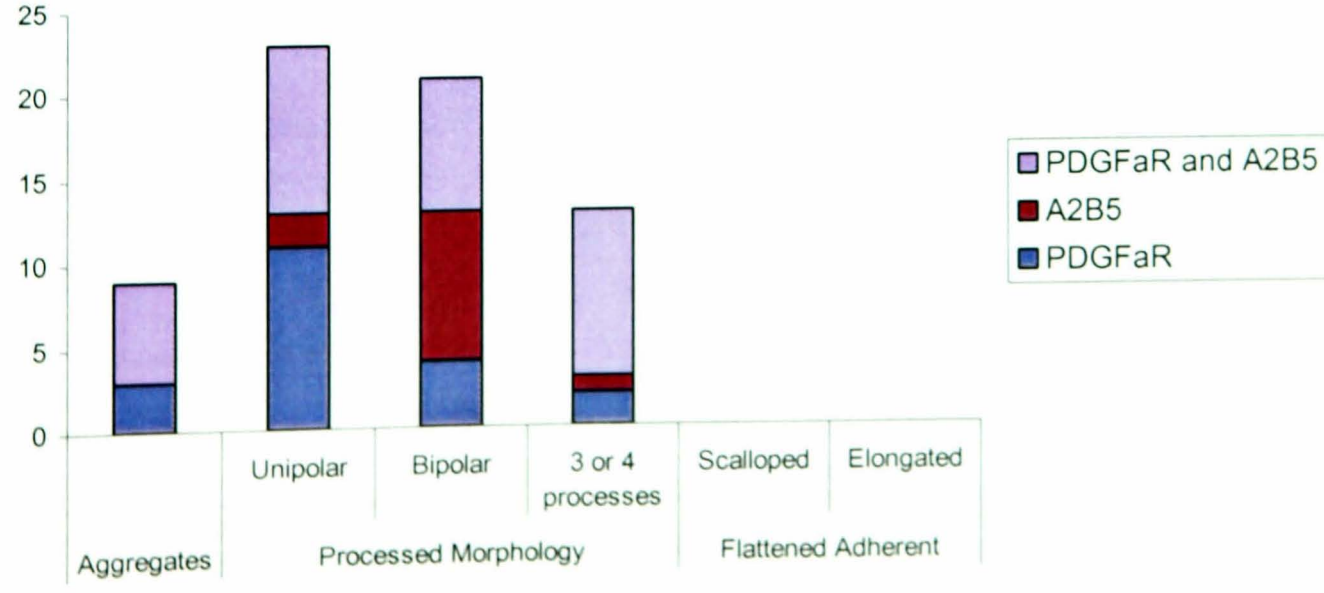


Figure 4-6 PDGFαR, NG2 and A2B5 antigens are expressed on an overlapping population of cells [Representative analysis of a single coverslip – Immunophenotype of 106 cells out of a population of 989 recorded]

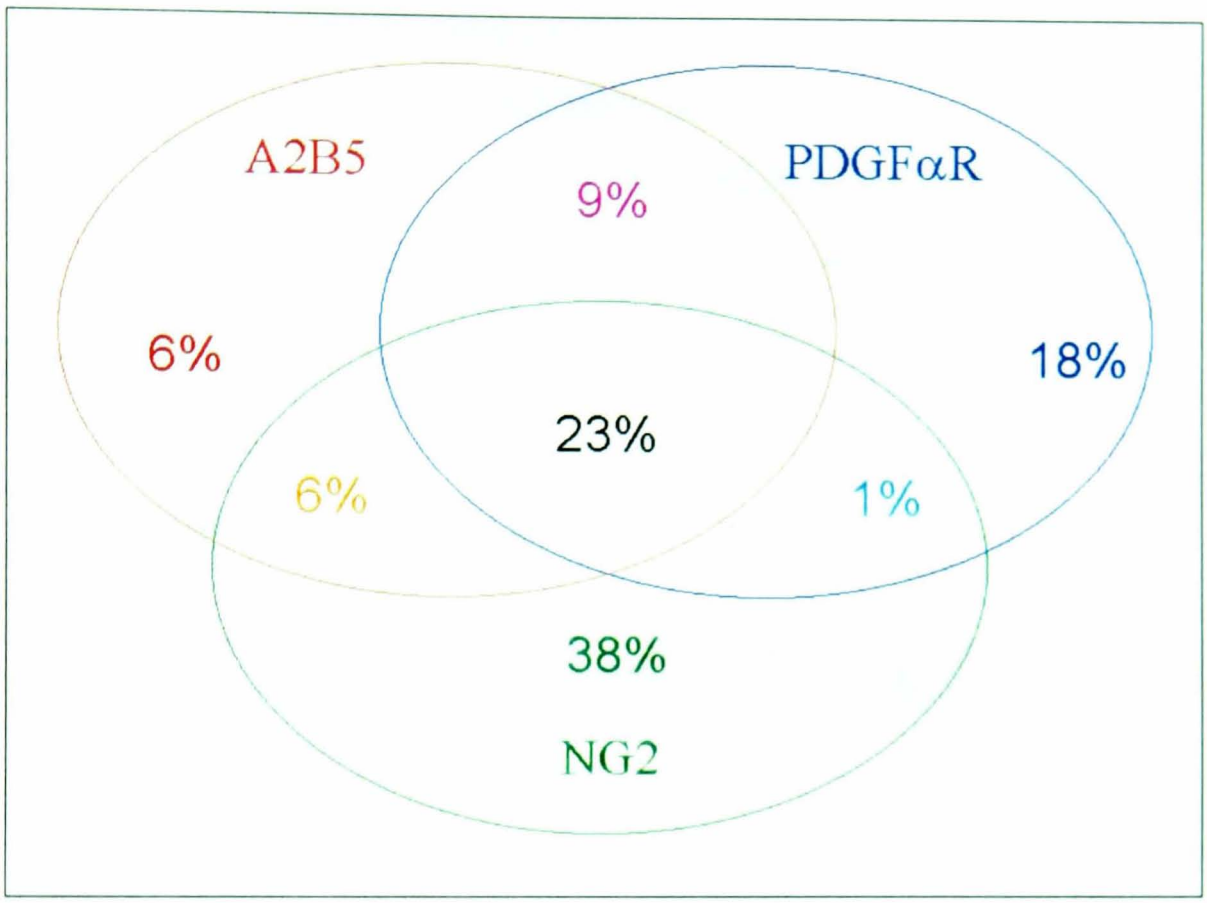


Figure 4-7 One hypothesis to explain the relationship between cells labelled with PDGFαR, A2B5 and NG2

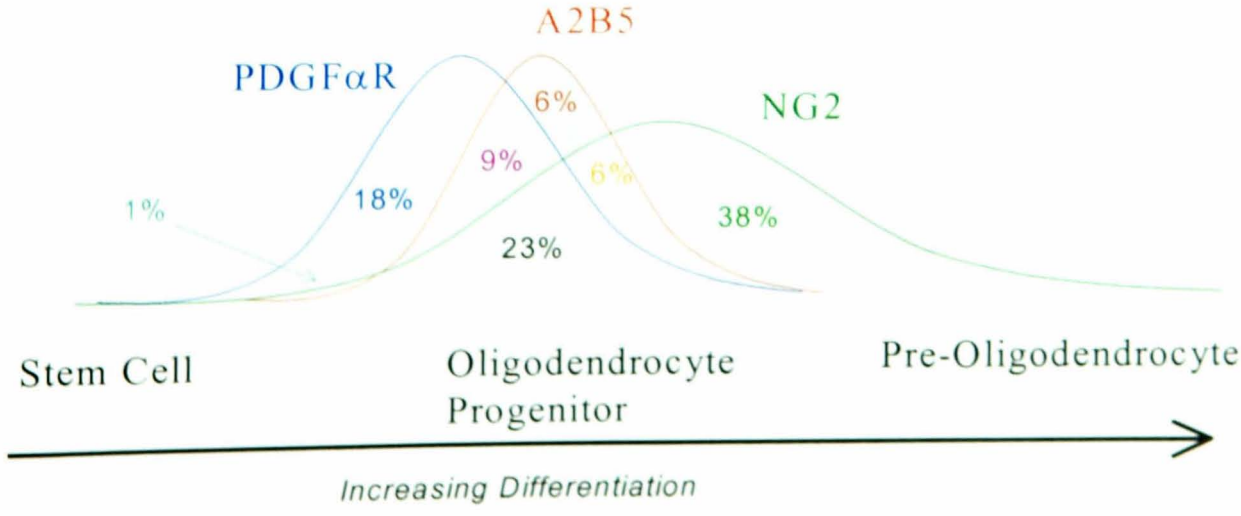


Figure 4-8 Acquisition of antigen markers and morphological complexity as cells mature through the oligodendrocyte lineage

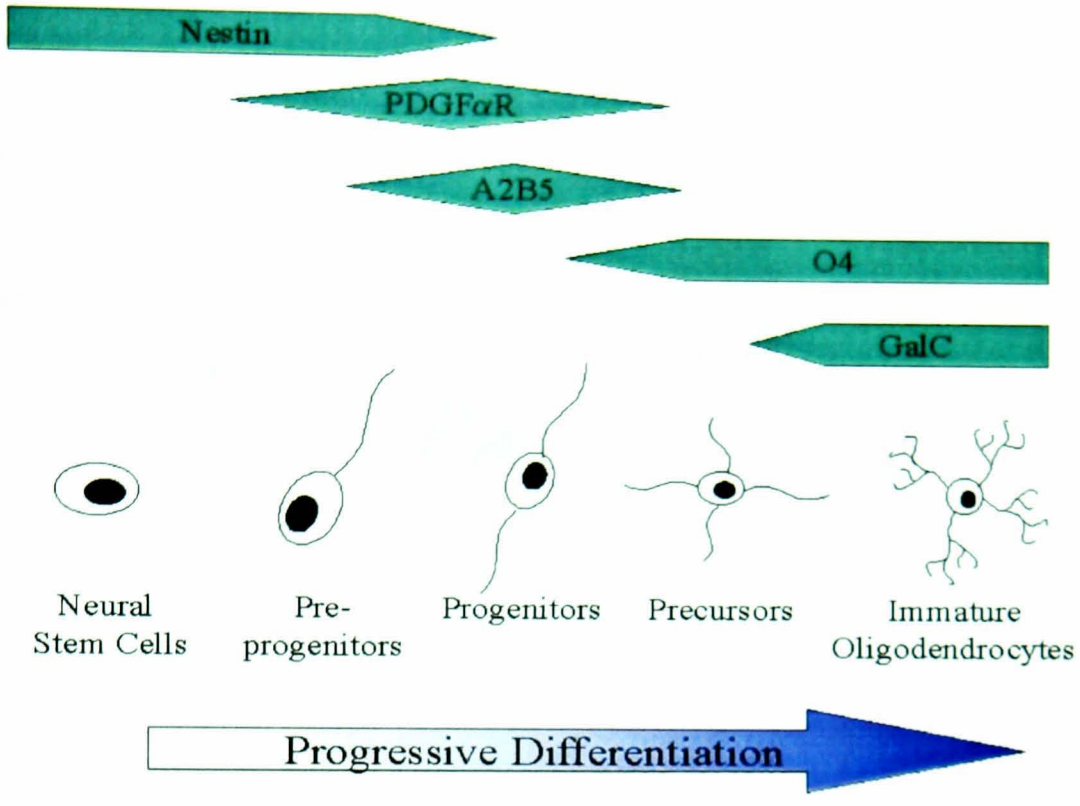


Figure 4-9 Morphology of various immunophenotypes of putative OPCs [Immunophenotype of 245 cells recorded from a single representative coverslip of >1000 cells]

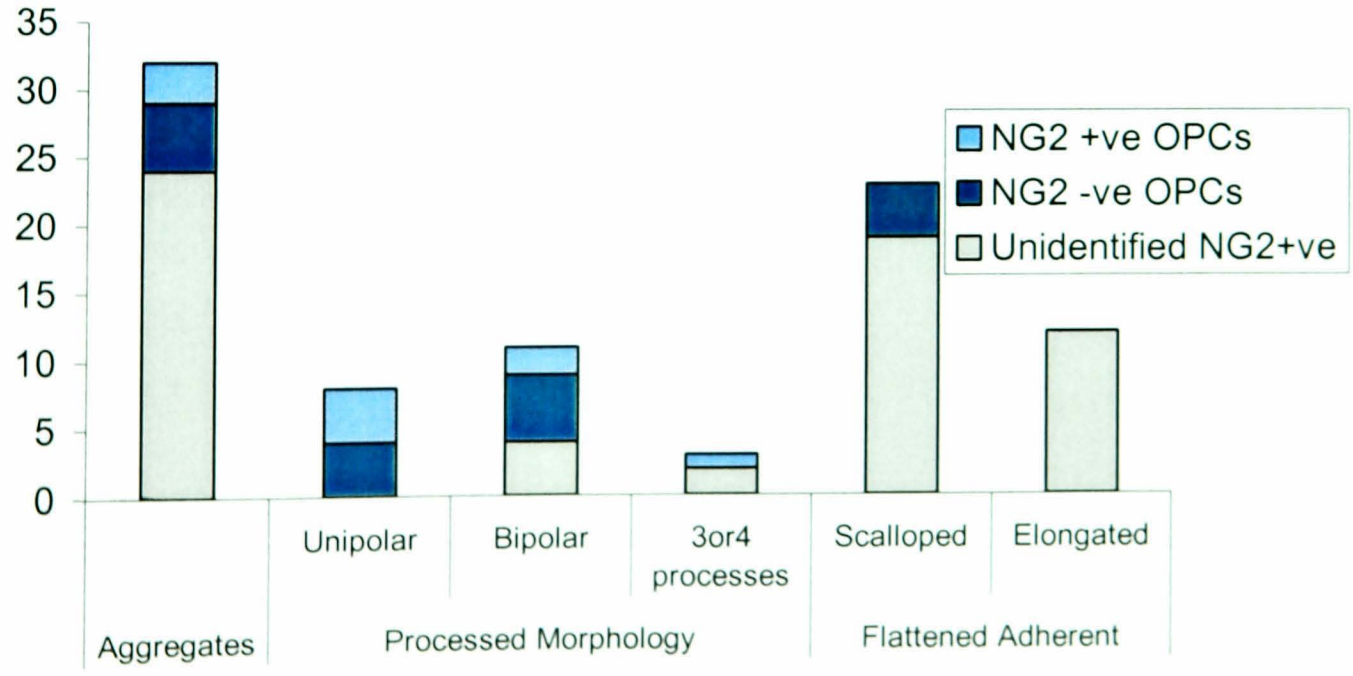


Figure 4-10 Proportion of total cell numbers staining with early glial markers [1819 cells counted from a single coverslip]

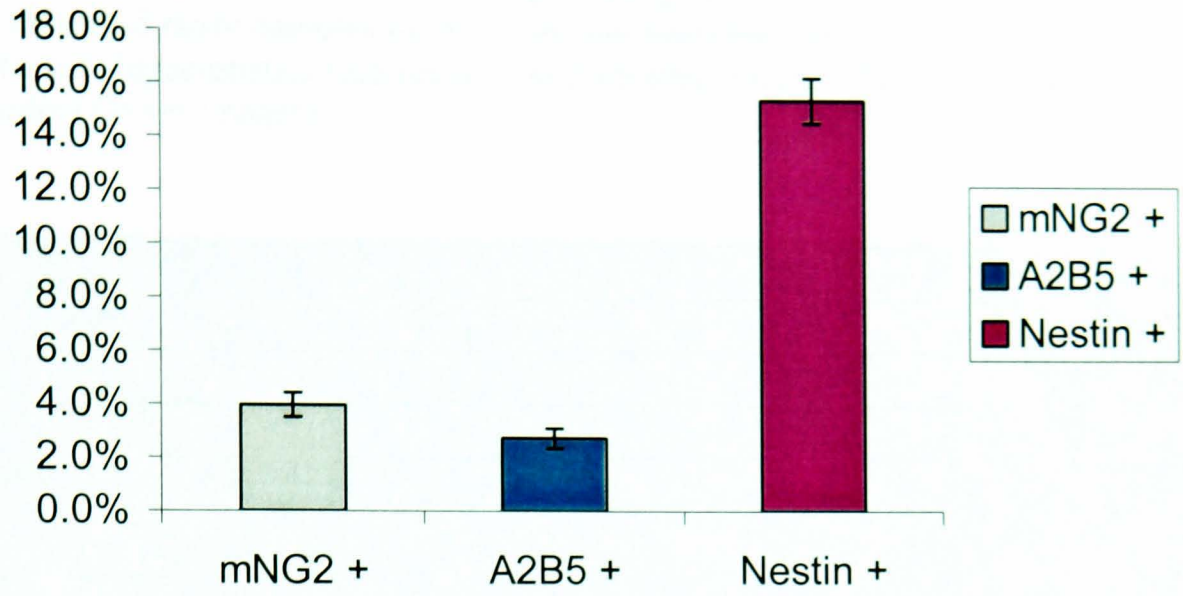


Figure 4-11 Proportion of NG2+ cells staining for nestin and A2B5 on a single representative sample at 5 DIV [72 NG2+ cells counted from one coverslip of 1819 cells]

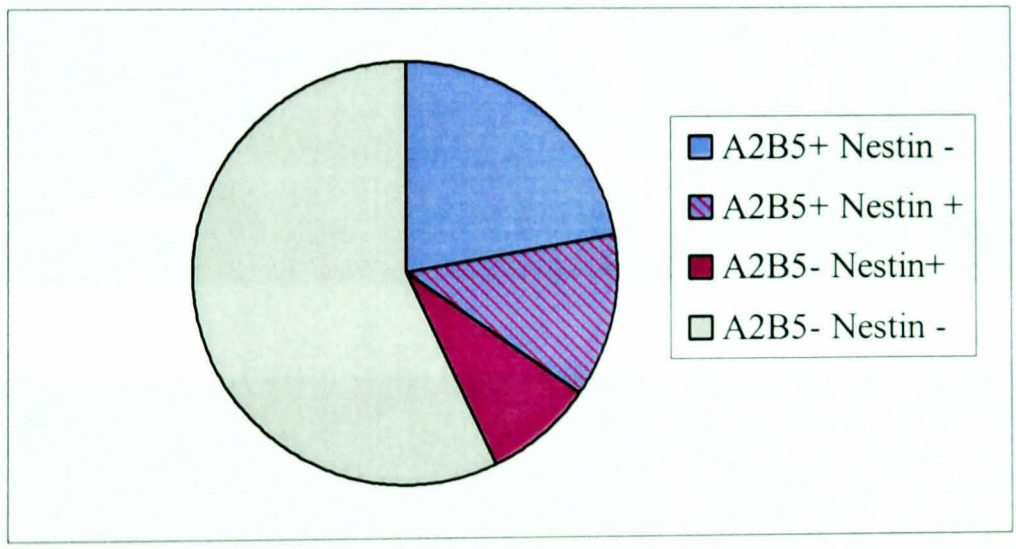


Figure 4-12 A number of NG2+ cells had a distinctive flattened appearance with widened processes, and did not stain for other glial antigens. (NG2 – FITC (green), Bar = 10μm). Some of these had fairly complex morphology that resemble oligodendrocyte lineage cells but retained characteristics features such as flattened, closely adherent processes and arcuate borders (lower images)

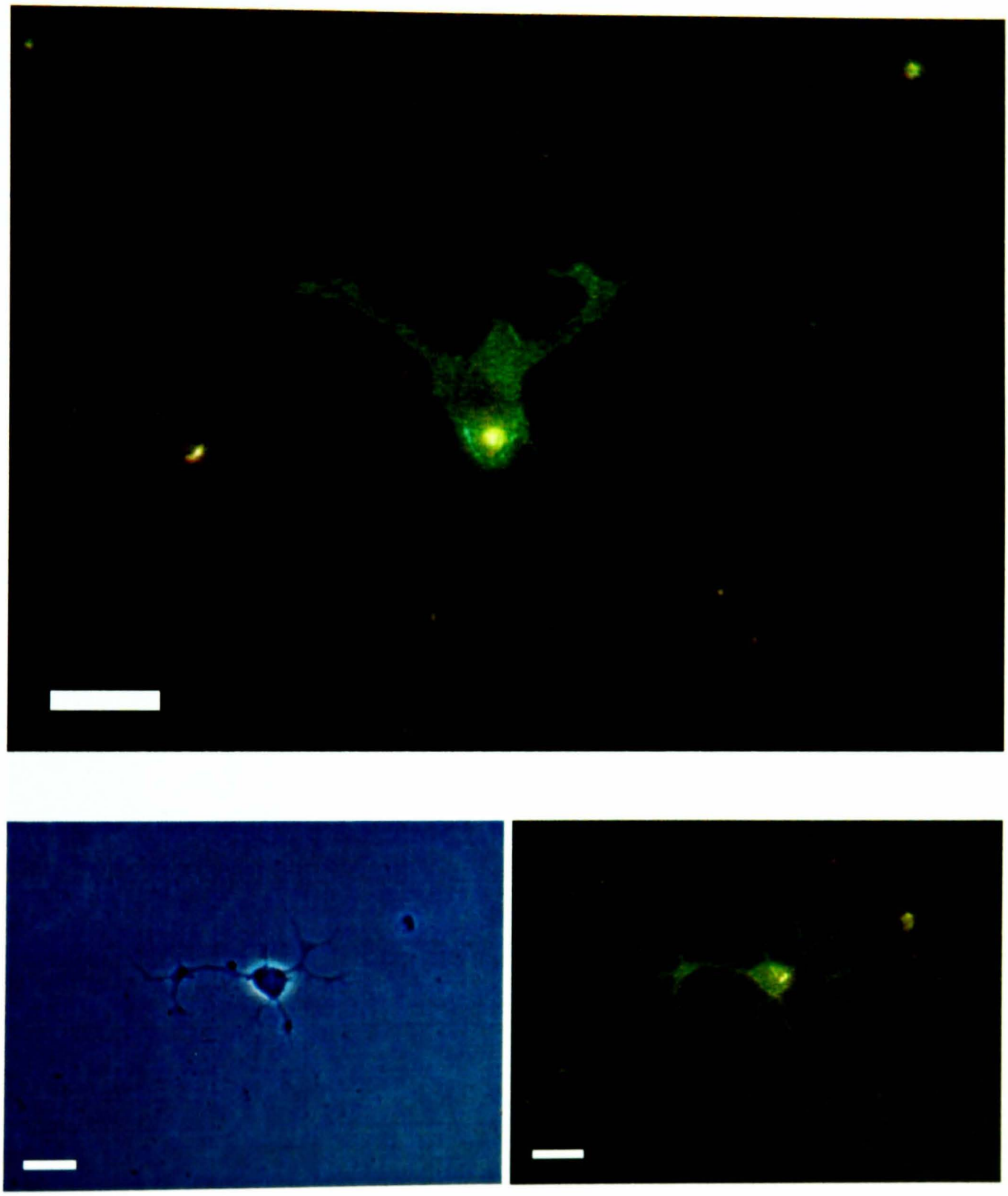


Figure 4-13 Fibroblasts in an adherent monolayer stain strongly with NG2 (green), but overlying microglia, identified by CD11b (red), show only yellow autofluorescence (Bar =20μm).

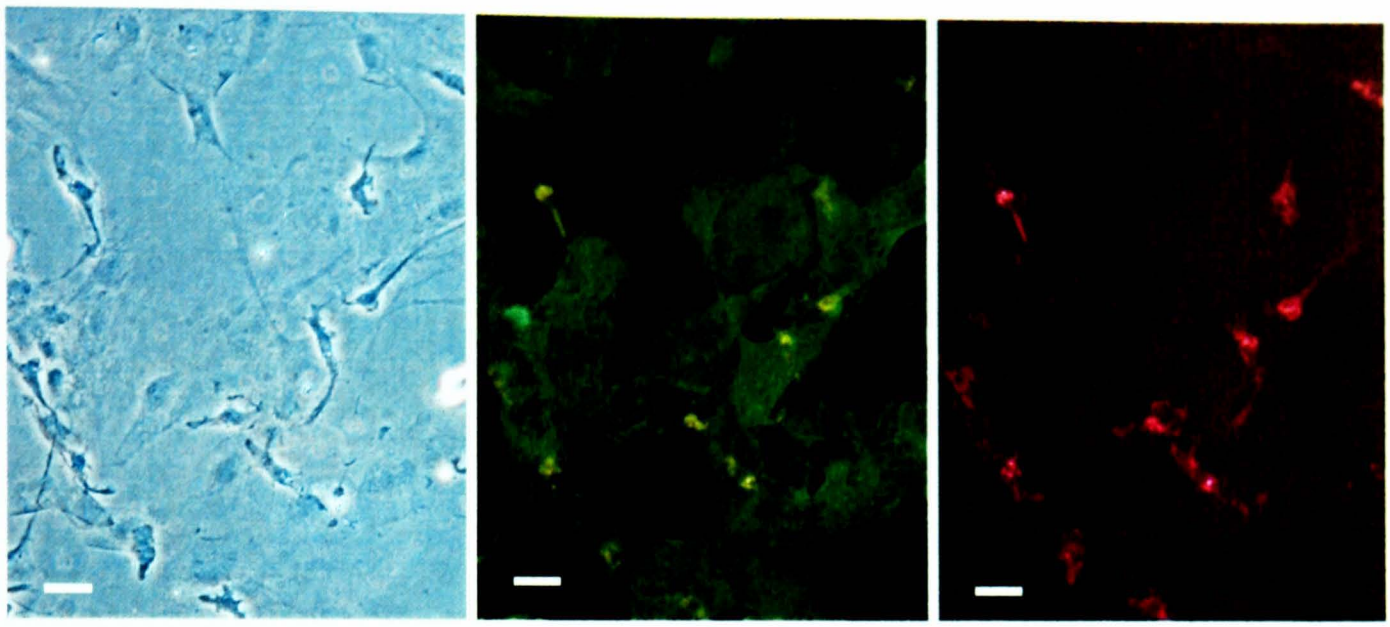


Figure 4-14 Microglia, stained with CD11b (red) occasionally stain very weakly for NG2 (green) *digitally enhanced image* (Bar = 10μm)

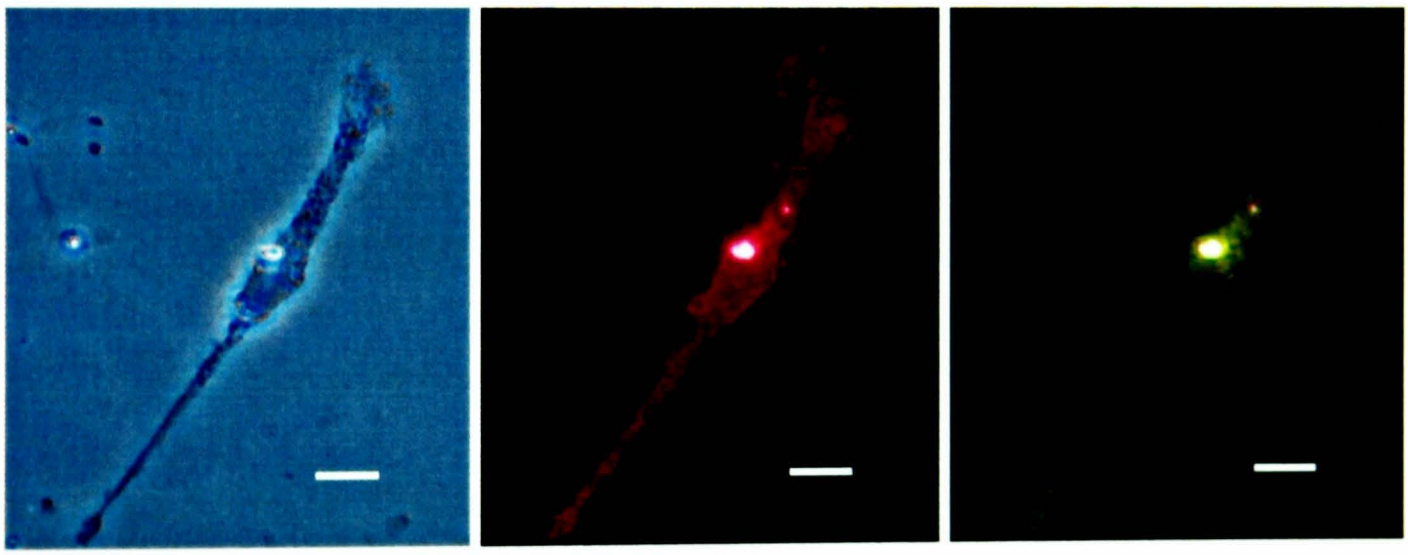


Figure 4-15 Identity of the NG2 positive population in mixed glial preparations [Composite graph showing cumulative percentages from multiple analyses (on separate coverslips) of a single human primary glial preparation]

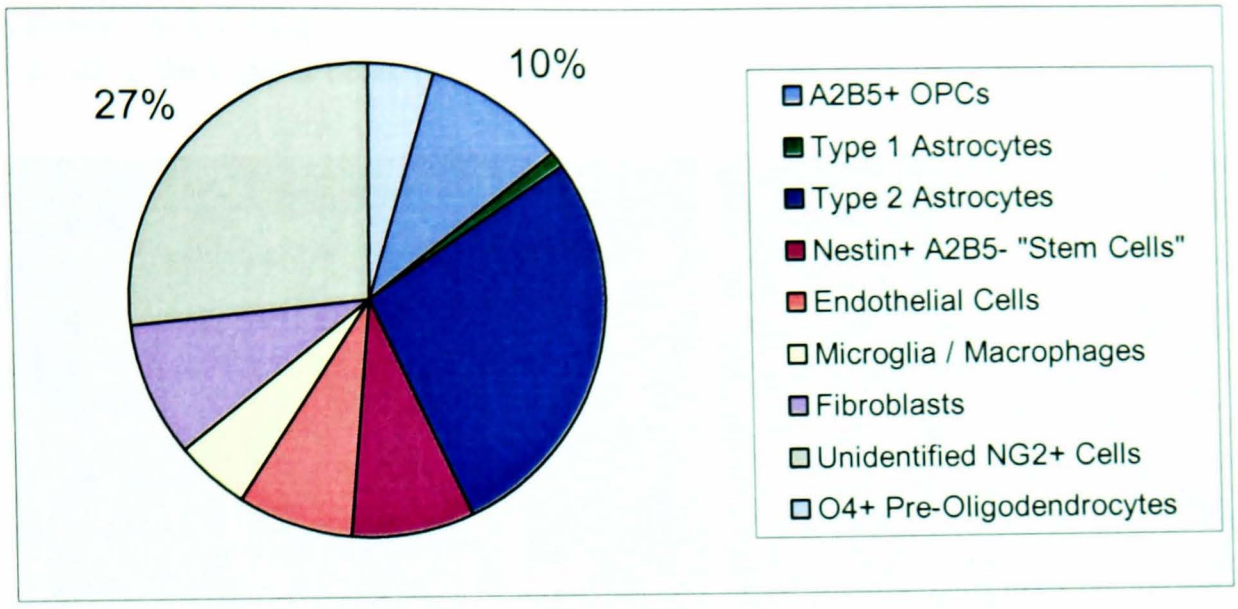


Figure 4-16 Cells separated by their expression of A2B5 antigens using magnetic beads develop into A2B5+(red) GFAP+(blue) type 2 astrocytes, and a number of small bipolar NG2+(green) cells (solid arrows) which appear to grow in clumps and have mostly lost A2B5 antigens. A few magnetic beads remain attached to the astrocytic cell bodies (hollow arrows) but not to the bipolar cells. (Scale bar = 20μm)

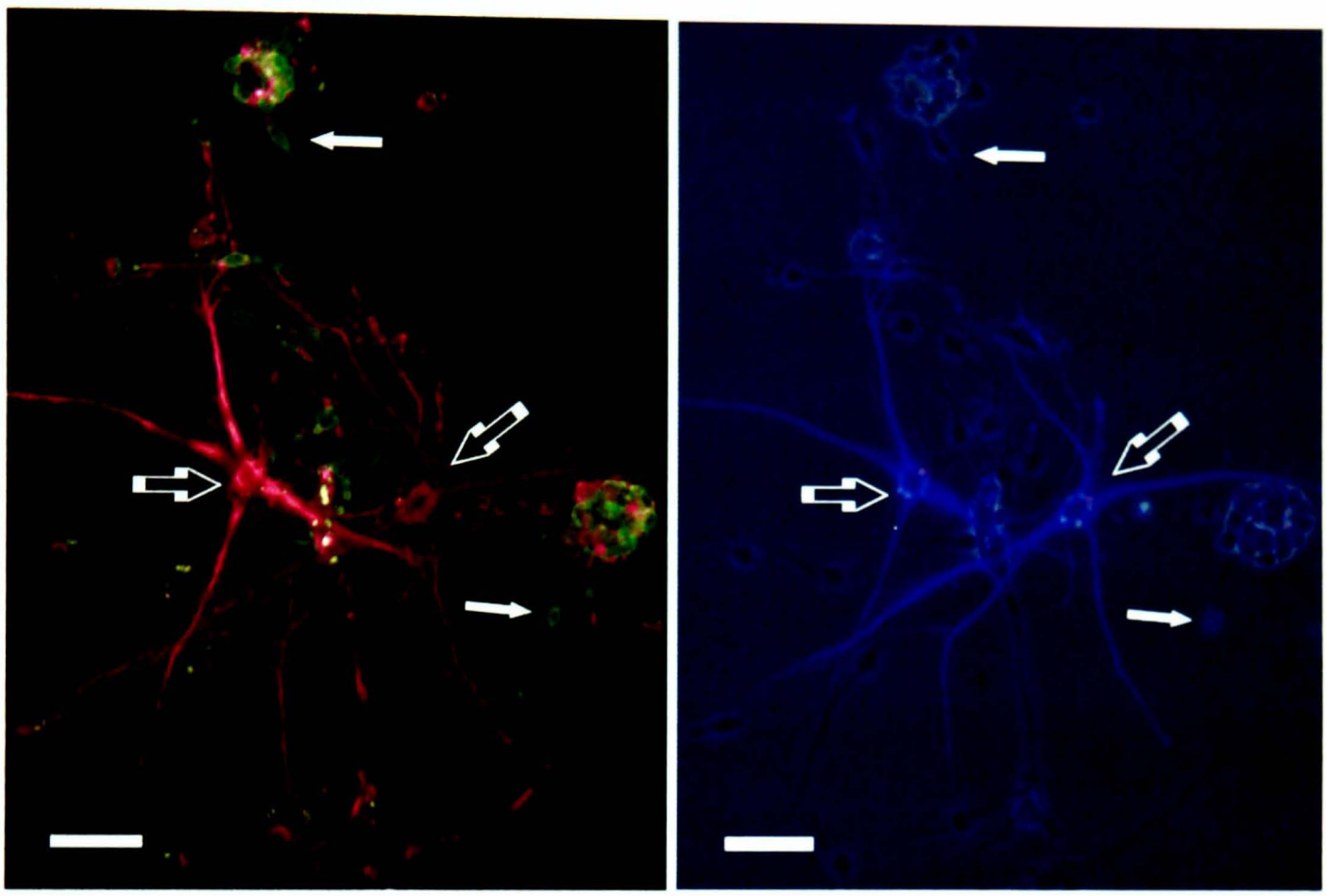


Figure 4-17 The same population of cells (A2B5, red & GFAP, blue) do not express neuronal antigens (β_{III}tubulin, green) (Scale bar = 20μm)

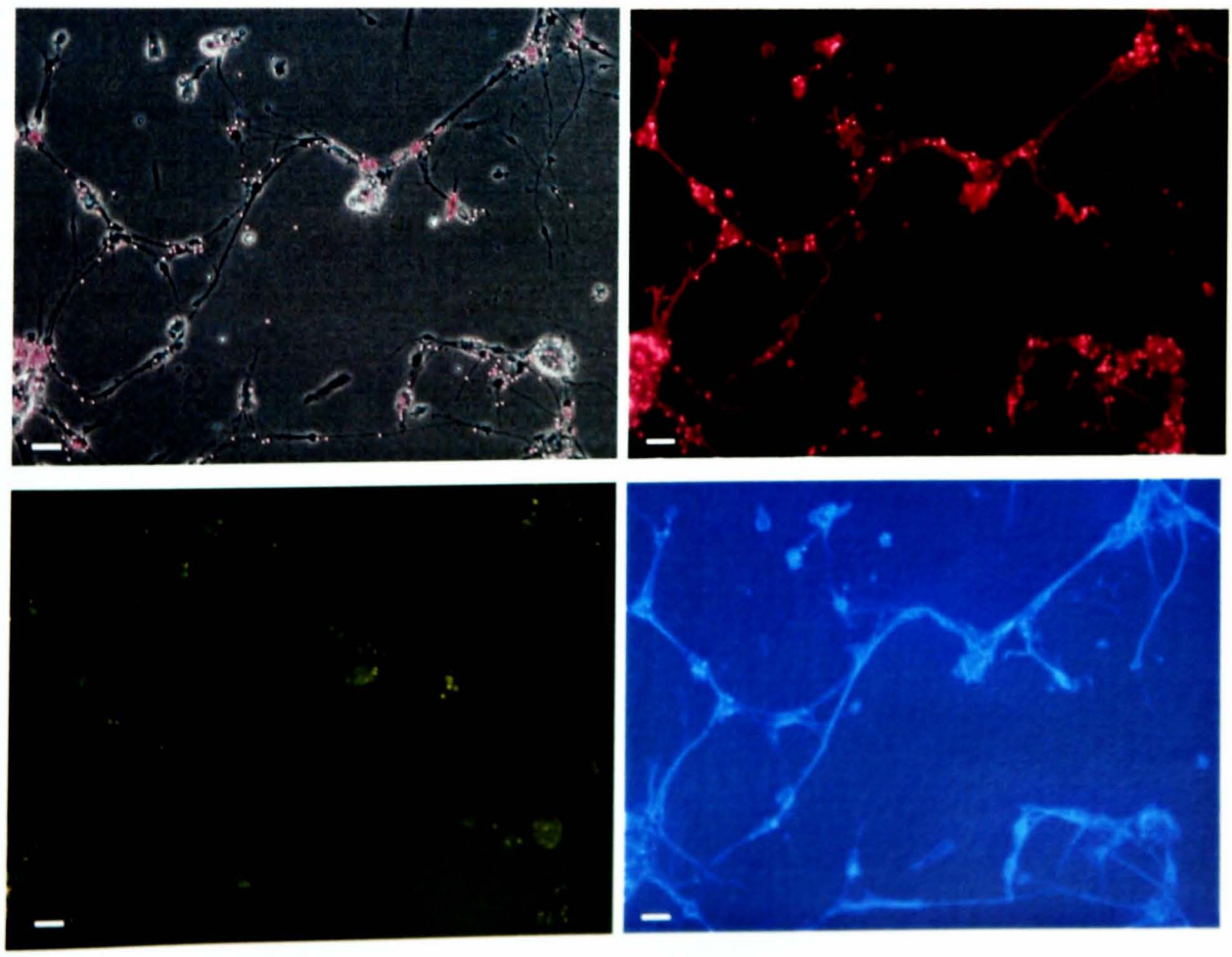


Figure 4-18 Cells selected for A2B5 using magnetic beads and expanded with mitogens. NG2 staining bipolar and clawed cells are visible, but only type 2 astrocytes retain A2B5 expression in this culture system

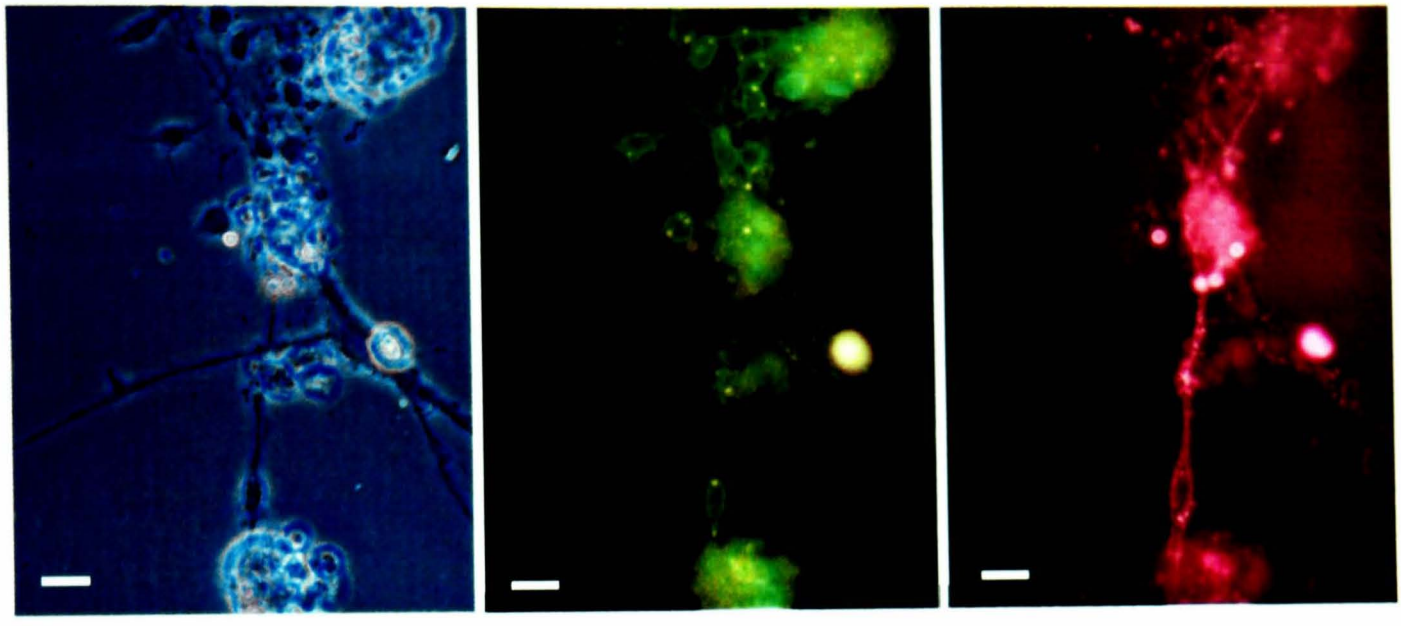
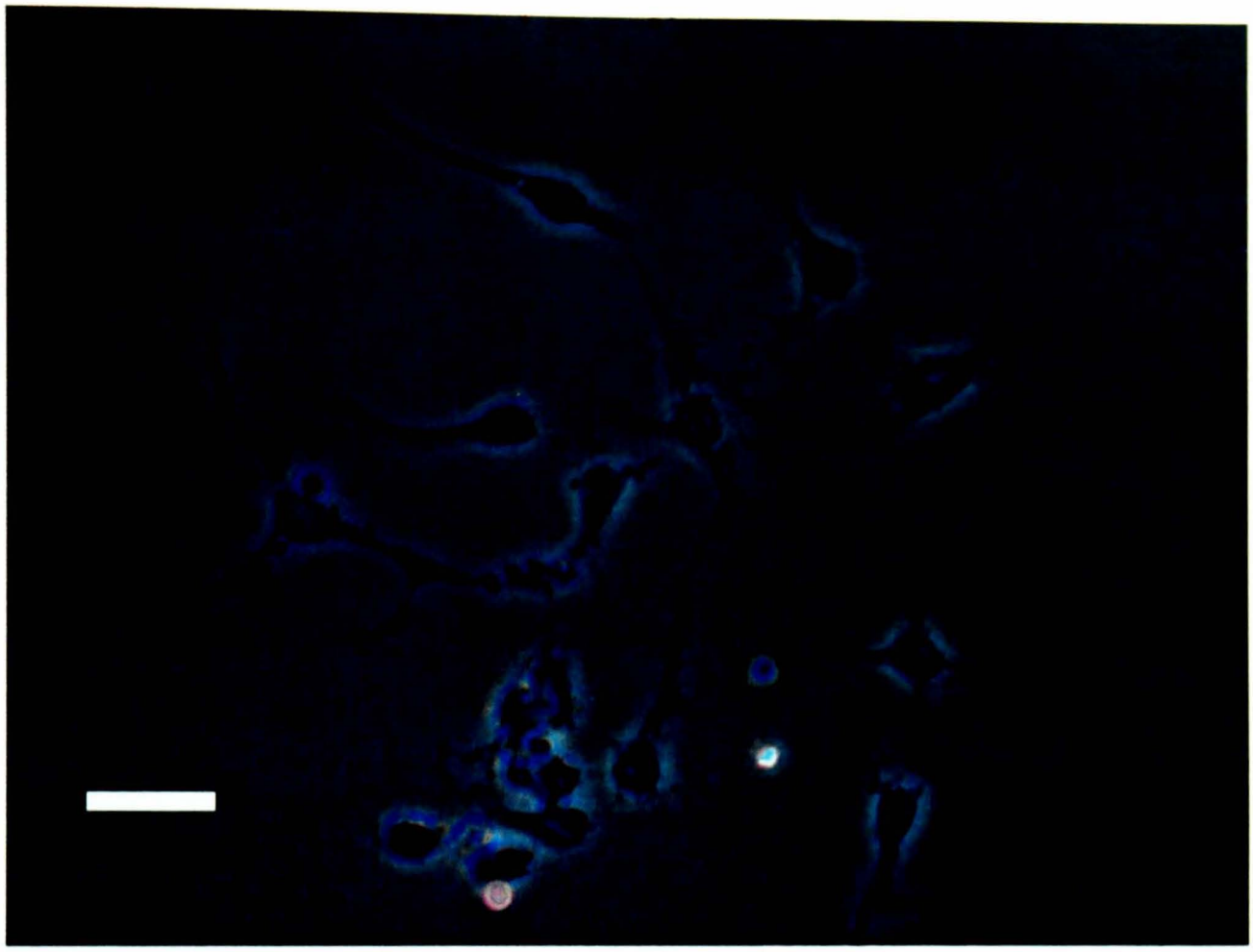


Figure 4-19 These cells, stained with NG2 (green) are morphologically diverse, and range from elongated unipolar and bipolar cells with wide, flattened processes, to more complex morphologies with the characteristic features of flattened adherent processes and arcuate, phase bright edges (bar =10 μm)



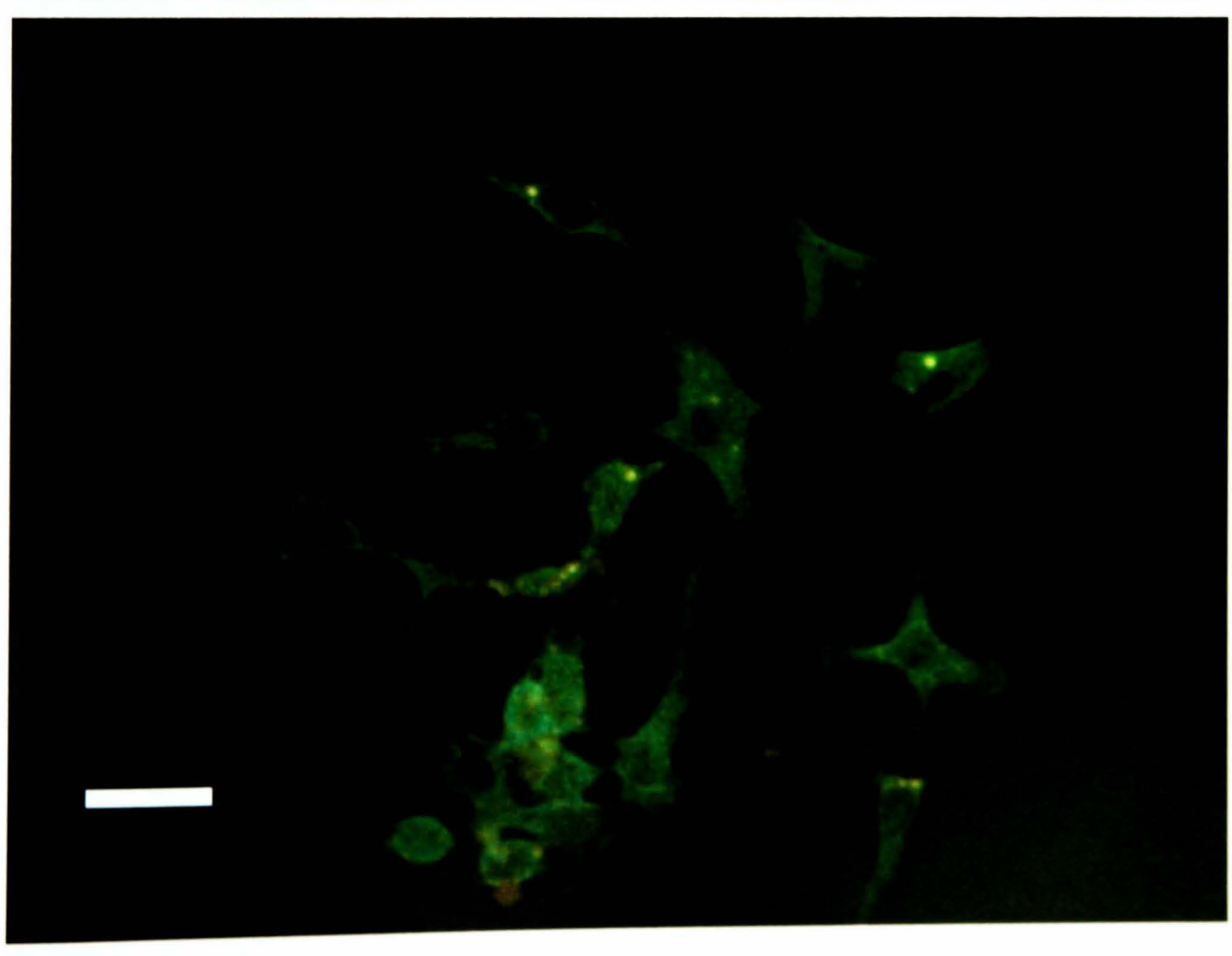


Figure 4-20 Dissociated cells selected for A2B5 expression and grown in FGF, PDGF and NT-3 express the PDGFαReceptor (green) and incorporate BrDU (red). (scale bar = 10μm)

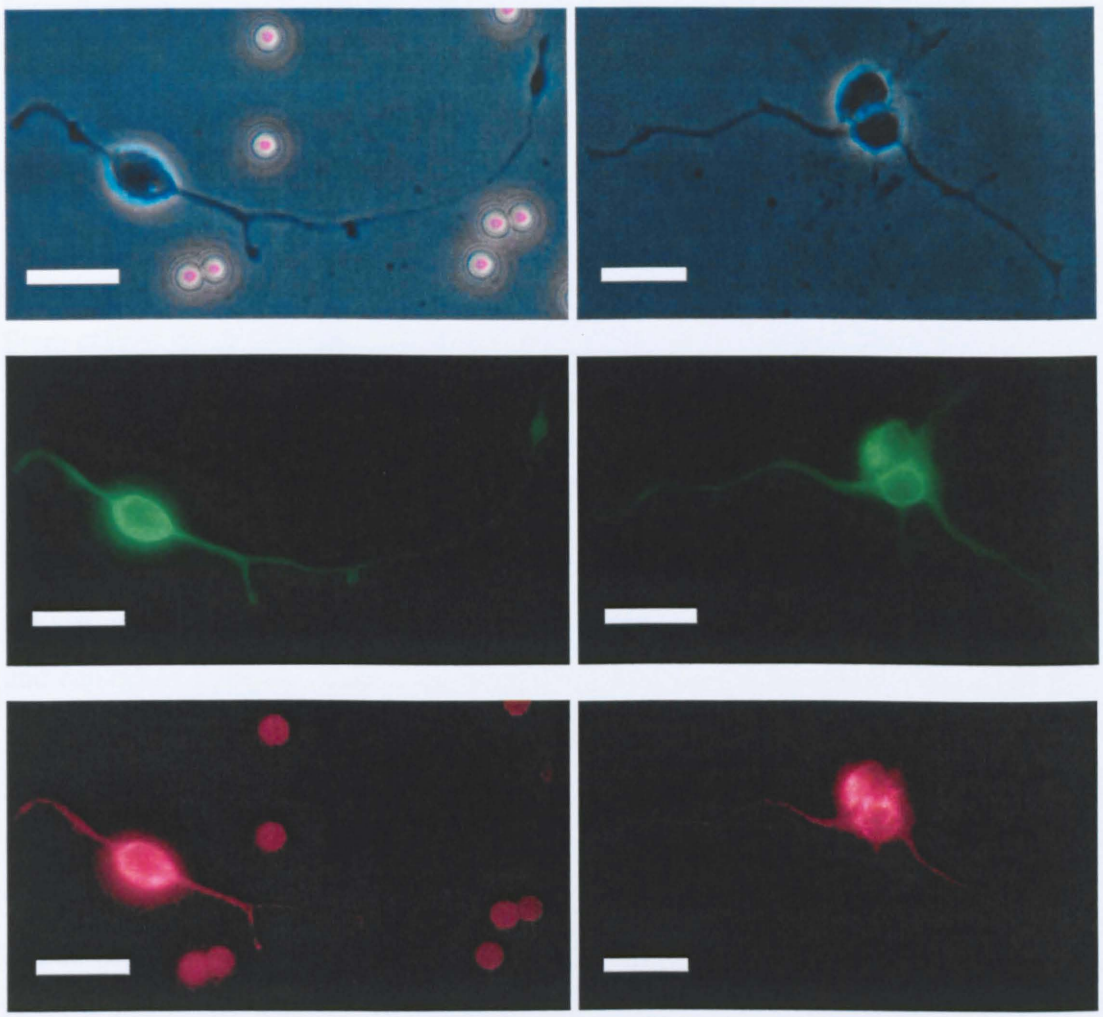
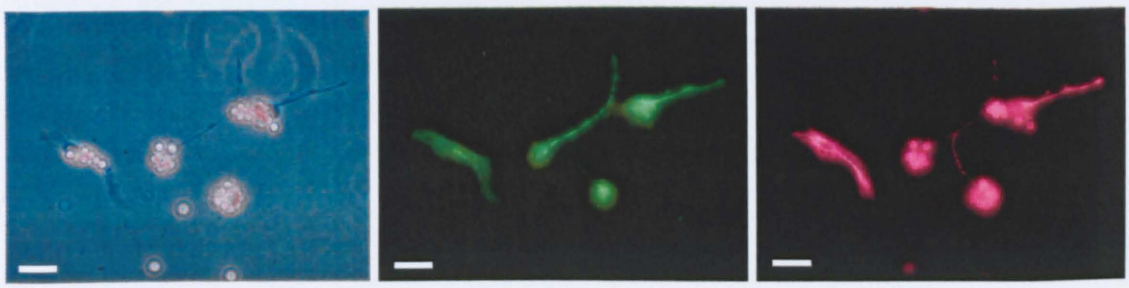


Figure 4-21 A2B5 positive cells selected out by magnetic beads, grow on poly-D-lysine and vitronectin, and develop 1-3 thin processes. They express both the PDGFαReceptor (green) and Vimentin (red).



Chapter 5. Neural Stem Cells as a Source of Human Glial Progenitors

Introduction

Attempts to immortalise human progenitor cells artificially were discussed in Chapter 2. The possibilities of expanding committed progenitors to provide large numbers of these cells, or inducing dedifferentiation to produce less committed (and hence more proliferative) cells was described in Chapters 3 and 4. Here the tactic of obtaining neural stem cells directly from adult CNS tissue is explored.

Stem cells possess an intrinsic ability to produce a huge number of daughter cells, and as such hold significant promise as a solution to the problem of obtaining sufficient cell numbers for systematic experimentation and ultimately therapeutic remyelination. They do this, at least in part, by dividing asymmetrically, so producing both an identical copy of themselves and a more committed progenitor. Their other properties are also of great interest to the neuroscientist. They are by definition pluripotential, and can act as a source of any cell intrinsic to (or required for) the mature organ from which they were derived (in the case of neural stem cells; astrocytes, oligodendrocytes and neurons). Furthermore, the property of asymmetrical division offers the potential for maintaining a stock of precursor cells with essentially the same pedigree – of great value in research paradigms.

In addition, stem cells have great therapeutic potential. Rodent embryonic stem cells possess significant remyelinating potential (Brustle et al. 1999), but their equivalent human sources – aborted foetuses or embryos surplus to IVF requirements – are not easy to access. It is commonly suggested that stem cells from embryos cloned for the purpose (by cell nuclear transfer) from the patient needing an implant, as recently legalised uniquely in the UK, would have the huge advantage of avoiding rejection. However, the proposal that every patient requiring a transplant would first have to be cloned seems quite unrealistic, and the serious ethical and practical difficulties pertaining to all sources of human embryonic stem cells have stimulated the largely successful search for alternative sources (Scolding 2001). It is now clear that neural stem cells are also present in the adult rodent brain (Weiss et al. 1996) and large numbers of rodent oligodendrocyte lineage cells can be generated using neurosphere/oligosphere techniques (Avellana-Adalid et al. 1996;Rogister et al.

1999;Vitry et al. 1999;Zhang et al. 1998b;Zhang et al. 1998a;Zhang et al. 2000), and these, upon transplantation, successfully remyelinate axons (Zhang et al. 1999). It has been reported that neural stem cells are also present in the adult human brain (Johansson et al. 1999;Kukekov et al. 1999) and cells with many of these properties (albeit derived from tumour surgery specimens) produce peripheral myelin following transplantation (Akiyama et al. 2001a). This is in contrast to reports from other mammalian systems where prior commitment to the oligodendrocyte lineage was a prerequisite of successful myelin formation (Smith & Blakemore 2000).

We hypothesised that the adult human brain does indeed contain neural stem cells. Secondly, we proposed that stem cells from adult human brain, mitogenically expanded *in vitro*, could differentiate into oligodendrocyte lineage cells, and could ultimately be used as a source of cells for remyelination therapies.

In testing these hypotheses we anticipated that the first evidence of adult neural stem cells might be the appearance of free-floating spherical bodies in these cultures. Most reports of mammalian neural stem cells growing in culture describe the formation of enlarging aggregates, sometimes termed neurospheres (Chiang et al. 1996;Milward et al. 1997;Mokry et al. 1996;Weiss et al. 1996). While not exclusively seen with stem cells (primitive oligodendrocyte progenitors have been reported as growing in "oligospheres" (Avellana-Adalid et al. 1996;Decker et al. 2000;Vitry et al. 1999;Zhang et al. 1998b;Zhang et al. 1998a)) the demonstration of aggregate formation would be suggestive of their presence.

If sphere formation could be demonstrated, the next aim would be the demonstration of continued propagation in culture driven by mitogens reported to be pertinent to stem cell growth, such as EGF (Reynolds & Weiss 1996) and FGF2 (Kalyani et al. 1999). Stem cells have a notable replicative potential, not least by reason of their asymmetric division. Thus they would be expected to continue to expand under the influence of appropriate mitogens, and the production of appreciable numbers of cells from a limited source should be demonstrable.

Thirdly, mitogen-expanded adult human neural stem cells growing in spheres would be expected to stain for primitive neural antigens. Vimentin, an intermediate filament protein, is expressed by a number of primitive neural lineages, and distinguishes neonatal from adult rodent oligodendrocyte progenitors (it is only expressed by the former) (Wolswijk & Noble 1989). S100, a protein associated with intermediate filaments, appears to be expressed by immature astrocytes as well as labelling

Schwann cells. These antigens are expressed widely amongst primitive cells of various lineages (Richter-Landsberg & Heinrich 1995). Another intermediate filament protein of great interest is nestin, which is expressed in neuro-epithelial stem cells (Reynolds et al. 1992;Tohyama et al. 1992). This protein was also expressed in some of the earliest descriptions of stem cells derived from embryonic rodents and grown in culture (Reynolds et al. 1992). Furthermore, cells selected for high expression of this protein appear to be multipotent and sphere forming (Kawaguchi et al. 2001). While not all authorities agree as to immunophenotype of the resting, and hence possibly rather rare, adult neural stem cell (Doetsch et al. 1999;Morshead et al. 1994) reports of neural stem cells growing as spheres usually report nestin expression (Johansson et al. 1999;Reynolds et al. 1992), with occasional exceptions (Kukekov et al. 1997).

Fourthly, individual spheres formed by true neural stem cells would be expected to give rise to cells of all three neural lineages. In particular, the demonstration of cells bearing neuronal antigens would have particular significance, as adult neurones are not expected to survive the dissociation procedure.

Unequivocal demonstration of neural stem cells requires rigorous clonal analysis. While this is undoubtedly desirable, it is time consuming and is not a direct prerequisite of a potential cell source for therapeutic remyelination. Of considerably more pertinence to this cause is the demonstration of successful commitment to the oligodendrocyte lineage. The demonstration of functional remyelination following transplantation would be a long-term goal.

Methods

Adult human stem cell cultures

Several methods were employed for dissociating stem cells from surgical specimens. Many of these were based on the protocol used for human oligodendrocyte precursor culture (Armstrong et al. 1992) with certain modifications (*vide infra*). However other protocols were used in the early stages of this work, including that used by Kukekov et al in their work on human and murine stem cells (Kukekov et al. 1997;Kukekov et al. 1999). The latter will be described briefly, but the yield was so poor that it was not pursued further.

Most human tissue was provided from temporal lobe resections for intractable epilepsy. (It is worth re-emphasising that comparable tissue, dissected from sites distant from the epileptic focus, was histologically normal (Scolding et al. 1995)). Occasional samples from frontal lobe tissue were made available during the early stages, while significant changes to the methodology were underway. The yield from these was poor – although it is unclear whether this was due to methodological variations in the dissociation protocols, surgical factors or due to intrinsic differences in the tissue itself.

All patients gave their consent for resected tissue to be used for research purposes, and ethics approval was obtained from both local and regional ethics committees. Tissue was transported to the laboratory at 4°C in Hibernation medium (Hibernate™ A, BrainBits, Southern Illinois University) supplemented with penicillin / streptomycin / fungizone 1% (Invitrogen). Tissue was processed between 1 and 12 hours post resection.

Dissociation

Tissue was placed under a tissue culture hood, and meninges and large blood vessels were resected. The tissue was then minced with a scalpel and digested. Tissue was incubated at 37°C for 20 minutes in 5 ml Enzyme solution 1 (containing MEM (Sigma M7278), 0.116mg/ml trypsin (Sigma T4424), 0.184mg/ml collagenase III (Lorne LS 4208) & 18.4μg/ml $DNAseI_{IV}$ (Sigma D5025)). The mixture was then centrifuged at 200g for 5 minutes, the supernatant replaced with a further 5ml enzyme solution 1 and incubated for a further 20minutes. Following centrifugation the supernatant was then

replaced with 5ml enzyme solution 2 (containing MEM, trypsin-EDTA (Sigma T3924) 23 & 9.2μg/ml respectively, 0.184mg/ml collagenase III & 18.4μg/ml $DNAseI_{IV}$) for 15 minutes at 37°C and centrifuged. Finally the supernatant was replaced with 1ml of enzyme solution 3 (containing MEM, 2.4mg/ml trypsin inhibitor (Sigma T6522), 1.9 μg/ml $DNAseI_{IV}$, 1.6mg/ml bovine serum albumin (Sigma A4919) and 5% heat inactivated fetal bovine serum (Sigma F4135)) and triturated three times through 21 gauge and 23 gauge needles.

Trituration and Filtration

The original protocol favoured the use of three needles, 21, 23 and 25 gauge respectively and three triturations through each needle. Careful assessment of cell numbers and viability using differential nuclear staining with Hoechst and propidium iodide dyes (see below) showed that the increase in total cell numbers released by the final 25 gauge trituration was accompanied by a net fall in the viable cell yield. Again this was omitted from the later preparations.

To identify free-floating aggregates required the certain removal of clumps of undissociated brain tissue. This required a filtration step. Two filters were used, a 40μm stainless steel mesh and a 20μm nylon mesh, both housed in a Nalgene filter (Millipore, UK). During the early demonstration of aggregate culture, both were used sequentially. However, once confirmed that these cells were reliably growing from fully dissociated cultures, the 20μm mesh was omitted, as this resulted in a significant drop in cell yield. Following trituration, the mixture containing cells, debris and enzyme solution 3 was passed through the filter and flushed with 20ml 10% FCS in DMEM.

Gradient Centrifugation for Myelin Removal

Various options were tried for removal of myelin debris. This included the sucrose gradient centrifugation step used by Johansson et al in their early demonstration of adult human neural stem cells (Johansson et al. 1999), which was partly successful, although a large number of cells were lost. Optiprep™ was found to be more successful and a final concentration of 9% was determined as optimal. This was used for all the later preparations.

The (filtered) cell mixture was spun down and the supernatant aspirated to near dryness. The cells were then resuspended with 9%Optiprep in DMEM and spun at

2200rpm for 20 minutes. This formed a surface layer of myelin debris, which was easily aspirated off, a supernatant containing mostly cell debris and few viable cells, which was also removed, and a bottom pellet of cells and red blood corpuscles. The latter rarely interfered with the cultures or cell identification so no further methods for their removal were applied.

Alternative Dissociation Protocol (Kukekov et al. 1999)
Prepared and minced tissue (*vide supra*) was washed by adding 10 ml medium (DMEM with 1% PSF) and spinning down at 800 rpm for 5 minutes. Tissue fragments were resuspended and 5 ml 0.25% trypsin solution with EDTA was added. This was warmed in the hand, while gently aspirating and expelling through a 5 ml plastic pipette, to avoid the exuded DNA sticking the cells together. This process was continued until the supernatant was cloudy with cells, and the majority of the clumps were dispersed. The solution was then filtered through sterile gauze, prepared in a glass funnel. Viable cells were counted by Trypan blue exclusion in a haemocytometer. The remaining cells were centrifuged at 1000 rpm for 5 minutes and resuspended in DMEM/F12 with N2 supplements, FGF2 and EGF at double strength, with a final cell concentration not exceeding 100,000 cells per ml (minimum 6ml). This was then added to a 2% solution of methylcellulose (Sigma) in DMEM/F12 prepared beforehand, and mixed by adding to the top, and sucking from the bottom. 2ml of the resulting viscous solution was plated out into each well of a 6-well plate pre-coated with Poly HEMA anti-adhesive (see below) and incubated at 37°C in 7% CO_2.
In this protocol, 50 µl of a 40x solution of growth factors were added twice a week to each well.

Performing a Cell Count
Two methods were used. Trypan blue exclusion, using a 1:10 dilution of cell suspension in the dye was ideal for counting cells where there was little debris and few red cells. Dead cells and debris clumps are unable to exclude the dye and become stained dark blue, enabling them to be distinguished from the phase bright viable cell population. Hoechst and propidium iodide, both used at a final concentration of 10µg/ml, enabled both dead cells and viable cells to be independently counted despite considerable debris. Hoechst 33258 is permeant, so both viable and dead cells

fluoresce blue under UV light. Propidium iodide, which is impermeant, only labels cells with disrupted membranes, which fluoresce pink, a colour which overrides that of hoechst when visualised on the IX-70 immunofluorescent microscope. Glasstic™ counting chambers were used during cell dissociations as they facilitated the rapid assessment of sequential samples.

Coating Coverslips with Polylysine and Vitronectin

13mm diameter glass coverslips were added to 100% Methanol in a 50 ml Falcon tube, mixed thoroughly and left to stand overnight. The coverslips were then removed and layered between filter paper in a glass Petri dish, autoclaved and dried. They were then tipped into a 0.1mg/ml solution of poly-D-lysine, made freshly with sterile ddH_2O, in a Petri dish, mixed and left to stand for 1 hour. The coverslips were then removed individually, washed twice in sterilised ddH2O, and then leaned up against the side of each well in a 24-well plate. Any extra water was aspirated using a Pasteur pipette and a fine Gilson tip. The plates were then left overnight to dry.

Coverslips were either used with polylysine alone, or with another biologically active substrate in addition. 200µl of 1µg/ml vitronectin solution in ddH_2O was added to the centre of each polylysine coated coverslip and allowed to dry in situ.

Coating Flasks with Poly-HEMA™

Stem/progenitor cells are anchorage independent, and growing these cells in suspension selects them out from more differentiated cells. This can be achieved by using an anti-adhesive substrate such as Poly(2-hydroxyethyl methacrylate) - poly-HEMA™(Sigma, UK).

A stock solution of Poly HEMA™ was prepared by dissolving the product in 95% ethanol at 120mg/ml with vigorous shaking and overnight incubation at 37°C. Undissolved product was removed by centrifugation at 2500rpm for 30 minutes. Further dilutions of stock solution with 95% ethanol were occasionally used to produce coatings of various thickness – the greater the thickness, the greater the inhibition of attachment. Flasks were coated with 0.1ml/cm^2 of surface, and allow to air dry in a sterile environment for several days.

B104 Conditioned Medium for Human Stem Cells

B104 cells were grown on uncoated tissue culture plastic in 10% FCS in DMEM (*vide infra*), and passaged 1:10 approximately every 4 days. When approximately 70%

confluent, the medium was aspirated to dryness and cells were washed twice in Hank's balanced salt solution. This was then replaced with NSA-N2 with insulin and 2mM glutamine, and the incubation was continued for 3 days. The supernatant was then harvested, spun at 5500rpm for 1 hour, passed through a 0.4μm filter and frozen at –20°C until required.

The optimised solutions used in the final preparations were as follows:-

NSA-N2 based "stem cell mix" to make 50ml			*final concentrations*
NS-A medium	[Euroclone]	50 ml	(DMEM/F12+6%gluc)
N2 supplement	[Gibco]	0.5 ml	1%
L-Glutamine (200mM stock)		0.5 ml	2 mM (292mg ml)
Insulin (10mg/ml)		100µl	20 (total 25µg ml)
EGF	(200µg/ml stock)	5 µl	20 ng ml
FGF2	(100µg/ml stock)	5 µl	10 ng/ml
LIF	(10µg/ml)	10µl	2ng/ml

NSA-N2 based "OPC mix"			*final concentrations*
NS-A medium	[Euroclone]	50 ml	(DMEM/F12+6%gluc)
N2 supplement	[Gibco]	0.5 ml	1%
L-Glutamine (200mM stock)		0.5 ml	2 mM (292mg/ml)
Insulin	(10mg/ml)	100µl	20 (total 25µg/ml)
PDGFAA	(100µg/ml)	10µl	20 ng/ml
FGF2	(100µg/ml stock)	5 µl	10 ng/ml
NT-3	(10µg/ml)	10µl	2ng/ml

10% FCS in Sigma DMEM to make 200ml:-

DMEM (Sigma 6546)	200ml
L-Glutamine (final conc. 2mM)	2ml
FCS heat inactivated	20ml
PSF antibiotics	2ml

2x stock solution of Propidium Iodide and Hoechst

Hoechst 33258 (100mg/ml)	2µl
Propidium Iodide (1mg/ml)	200µl
Phosphate Buffered Saline	20ml

Results

Dissociated cells from adult human brain can grow as aggregates in culture.

The aim of the initial stage of this study was to confirm whether aggregate cultures could be grown from adult human neural cells. The starting points for this investigation were the reports of neural stem cells in cultures from the adult human brain (Johansson et al. 1999;Kukekov et al. 1999). The conditions used in these studies were reproduced as far as possible, building on the experience of mixed glial preparations used in this laboratory. Crude cell preparations were then grown in the presence of EGF, FGF2 and high insulin concentrations (Mozell & McMorris 1991) in uncoated tissue culture flasks.

This technique did produce small aggregates of cells that appeared to grow slowly over the course of several weeks. First visible as small irregular groups of 4-8 cells, these grew to form a spherical body with a phase dark centre after about 1 month *in vitro*. These features distinguished them from the irregular clumps of homogeneous debris also present in these cultures. After a few weeks in culture some spheres started adhering to the tissue culture plastic and cells started migrating in a radial fashion to form a small corona of adherent cells. However the large amount of myelin and cellular debris present in these cultures made further identification difficult.

To characterise these cells, the medium containing the suspended cells and aggregates was removed, the cells (and debris) separated by centrifugation, resuspended in fresh medium and plated onto poly-lysine for 1 week. Following this a larger number of cells started to adhere, not only from the visible clumps but also from individual cells either surviving in suspension, or released from the spheres when resuspended. These quickly formed a network of processes of predominantly astrocytic origin (as determined by GFAP staining), with evidence of both oligodendrocyte lineage cells (GalC) and bipolar cells with the immunophenotype of oligodendrocyte progenitors (A2B5+ GFAP-)(Figure 5-1). Most cells had an immunophenotype and morphology consistent with, if not always typical of, CNS glia (Figure 5-2). However, occasional cells were seen that stained for both GalC and GFAP antigens (Figure 5-3). These two antigens are widely used as markers for oligodendrocytes and astrocytes respectively. However Raff et al reported cells with both GalC and GFAP staining in 1983 in their seminal paper on the O-2A progenitor (Raff et al. 1983), which are also seen in adult

human progenitors (Scolding et al 1995). Raff et al.'s hypothesis of aberrant differentiation induced by the culture conditions seems equally plausible here, although the significant possibility of occasional Schwann cell contamination could also explain this observation.

Aggregate cultures from adult human brain continue to grow as spheres in EGF and FGF2, and express immature neural antigens

To confirm that these cultures contained sphere-forming cells, a further glial preparation was established using the standard dissociation protocol. These cells were placed immediately into a DMEM:F12 medium with a high insulin concentration (sufficient to activate the Insulin-like Growth Factor (IGF) receptor), EGF and FGF2 and placed in an uncoated tissue culture flask. Growth factors were replaced 3x/week, and the medium changed 50:50 every week, ensuring that no cells in suspension were lost. The change to the base medium, and the replacement regime for growth factors were based on recommendations made by Dr V.Kukekov (personal communication) at around this time. A number of cells with a flattened, fibroblast-like morphology adhered to the tissue culture flask within days of plating and eventually formed a monolayer. However, after 7-10 days a few small aggregates appeared, and these developed into floating spherical bodies that continued to grow until the largest of these was approximately 200μm in diameter (Figure 5-4). This growth then gradually slowed despite continued growth factor replacement, until little change was seen after 3 months *in vitro*. Some of the supernatant and floating cells were removed at 12 weeks, concentrated and plated onto poly-lysine coverslips. These were left for a further week in the same medium to adhere before being stained for a number of immature and mature neural antigens.

Many of the cells that subsequently adhered to poly-lysine stained with vimentin or S100, but in view of their morphological similarities with "type 1" astrocytes the lack of widespread GFAP staining was surprising (Figure 5-5, Figure 5-6 & Figure 5-7). (Antibodies to human nestin were not available at this stage of the research.) Vimentin, an intermediate filament protein, is expressed by a number of primitive neural lineages, and distinguishes neonatal from adult rodent oligodendrocyte progenitors (it is only expressed by the former.) S100, a protein associated with intermediate filaments, appears to be expressed by immature astrocytes as well as labelling Schwann cells, but widespread expression amongst primitive cells means

neither of these antigens is regarded as being lineage specific (Richter-Landsberg & Heinrich 1995). However they provide suggestive evidence that these spherical aggregates contained a primitive population of cells.

To provide further evidence it was necessary to demonstrate the presence of all three lineages in these cultures. This was attempted using antibodies against neurofilament, GFAP, GalC and A2B5. The result of this experiment was unfortunately inconclusive. While there was a suggestion of fluorescent staining of the spheres with both A2B5 and GalC (Figure 5-6), the number of spheres grown in these cultures limited the staining protocol to 4 coverslips (3 triple immunofluorescent stains and 1 control), which was insufficient to rigorously exclude non-specific binding. Furthermore, although there was some staining of cells with the neurofilament antibody, these cells were morphologically unlike neurons, and the staining pattern was not indicative of cytoskeletal binding (Figure 5-6 and inset). Subsequent experiments demonstrated that more discriminatory immunofluorescence could be achieved with a lower concentration of anti-NF200kD antibody.

Cell proliferation and commitment to the oligodendrocyte lineage can be influenced by altering the culture conditions

In spite of the undoubted importance of determining the presence or absence of stem cells in these cultures, this was subordinate to the requirement of finding a source of oligodendrocyte progenitors. At this stage in the research there was a hiatus due to severely limited tissue supplies (only one sample became available between November 2000 and November 2001 due to medico-political fallout from the Alder-Hey Scandal). By the time this work resumed, several groups had published different protocols using different media combinations and it was unclear which would provide the largest yield of oligodendrocyte progenitors from our specimens. It was therefore decided to address this question directly by systematically investigating different culture conditions to optimise the yield of oligodendrocyte lineage cells from the aggregates.

Several reports had suggested that a high glucose concentration (6g/L) favoured stem cell growth over traditional DMEM:F12 formulations (3g/L) used in the earlier reports of stem cell culture (Carpenter et al. 1999). Most investigators had reported protocols including EGF and FGF2 as encouraging stem cell proliferation (Johansson et al. 1999) (although some suggest that the EGF-responsive and FGF-responsive

populations were separate (Martens et al. 2000)), but it was not clear to what extent these conditions had been obtained by systematic assessment. The hypothesis here was that the more primitive, putatively EGF- (& FGF-) responsive cells would proliferate best in these circumstances, giving high total cell numbers, of which a proportion would spontaneously commit to the oligodendrocyte lineage. However reports were emerging of EGF encouraging astrocytic differentiation at the expense of other lineages [S Goldman – personal communication]. Certain groups were using FGF2 in isolation to expand oligodendroglial precursors with some success (Grever et al. 1999). FGF2 appears to have mitogenic properties both in the stem cell population (Johansson et al. 1999;Kukekov et al. 1999) and in more committed progenitors (McKinnon et al. 1990) and it was hypothesised that this could be sufficient to expand stem cells without biasing their commitment away from oligodendrocyte development. Thirdly, the traditional combination of FGF and PDGF was selected because of its attested growth promoting properties on rodent oligodendrocyte progenitors. It was hypothesised that these mitogens would promote proliferation of oligodendrocyte progenitors as they differentiated from more primitive cells. The previous observations of oligospheres (free floating aggregates of proliferating oligodendroglial cells at the pre-progenitor and progenitor stage) had been reported to form in media known to promote the expansion of oligodendrocyte progenitors (Avellana-Adalid et al. 1996;Vitry et al. 1999;Zhang et al. 1998a). It was therefore possible that human progenitors might grow as free floating aggregates under these conditions, and to address this question it was decided to compare the cellular yield from both the adherent and floating populations.

To identify the optimum growth conditions, the next glial preparation was processed in 6 different ways (3 growth factor combinations by 2 glucose concentrations), and the yield from both the adherent and free-floating populations were assessed by immunocytochemical means.

The small number of cells available from a single brain specimen limited the statistical power of this experiment, and so comparisons between the groups were not significant. However, there was a trend towards an optimum yield with EGF and FGF in a high glucose medium (NSA – Euroclone™) (Figure 5-8). Roughly one third of these cells stained with A2B5, a higher proportion than seen in the other groups (again not statistically significant). Interestingly, very few adherent cells grew in these conditions, a further pointer to suggest that the final population of cells were

anchorage independent for at least some of their growth. These growth conditions were reproduced for subsequent experiments.

Aggregate cultures expand as spheres in stem cell mitogens

Further steps were taken to optimise the growing conditions and to demonstrate continued proliferation *in vitro*. The next sample was placed under similar growth conditions to those determined above, but three notable changes were made to the protocol. Firstly, a density centrifugation step was added to the dissociation protocol to remove most of the myelin debris. The continued presence of the debris made identification of free-floating cells problematic, and the loss of viable cells in this process was outweighed by the gains in terms of clarity and staining quality. Secondly, following advice from other groups, recombinant growth factors were replaced weekly rather than on alternate days. The final point related to the source tissue which was obtained from the convexity temporal cortex of a 4 year old boy with post meningitic epilepsy, while most previous samples had come from mesial temporal structures of young adults. These cells started forming aggregates as before, but the aggregates then started adhering spontaneously. As they plated down, they formed small nests of cells with a mixture of morphologies and further proliferation was halted (Figure 5-9). The immunophenotypes of these cells were not successfully identified, but it was felt that the paucity of growth factors might have been responsible for this.

To prevent this effect growth factors were once again added 3x /week during the next preparation. In addition, to inhibit premature adhesion and to remove the anchorage-dependant population, the tissue culture plastic was first coated with the anti-adhesive Poly-HEMA™ on parallel flasks. However the increased frequency of growth factor replacement alone appeared sufficient to reduce this effect, and total cell yield was notably reduced by the anti-adhesive coating. Spheres formed under these circumstances and continued to expand under the influence of EGF and FGF2 to sizes between 50-80μm in diameter by the third week.

Cells from these spheres express nestin and poly-sialated NCAM.

After an initial fortnight growing in suspension cultures and a four-day period on poly-lysine, to promote adherence and facilitate identification, a thorough delineation of cellular constituents was possible, supported by appropriate controls. Cells at this

stage adhered to the coated coverslips and migrated short distances from it. Most cells in the corona had a flattened or elongated, bipolar morphology.

Staining with an antibody to human nestin revealed widespread staining both of the migrating cells and of those cells still resident in the sphere. The cytoplasm stained in a pattern consistent with that expected for a cytoskeletal protein, and delineated processes clearly.

Glial and neural progenitors express the "embryonic" form of NCAM, which differs from the counterpart expressed on the surface of more mature cells by the presence of a chain of sialic acid residues. This chain may play an important role in migration by limiting cell-cell adhesion by means of its size (Murray & Dubois-Dalcq 1997;Vitry et al. 2001). Antibodies to PSA-NCAM clearly labelled the surface of many cells as they migrated out of the spheres onto the substrate(Figure 5-10).

Neural spheres contain cells from all three neural lineages

After a further three days on poly-lysine, these preparations were stained with markers specific for the separate neural lineages. Neurons were identified with the neuron specific β_{III} isoform of tubulin, astrocytes by glial fibrillary acidic protein and cells committed to the oligodendrocyte lineage with O4. Both neuronal cells and astrocytes were common in the adhering aggregates. Oligodendrocyte lineage cells were also evident at this stage but less prevalent (Figure 5-11 & Figure 5-12). It had previously been reported that neurons do not grow in primary cell preparations from adult human brain (Antel 2003). Several attempts using various markers have been made over 3 years to identify neurons in mixed glial cultures but we confirm that neurons do not survive the dissociation process. Thus the identification of cells expressing neuronal markers in these cultures after expansion of aggregate cultures in conditions known to promote stem cell propagation suggests that multipotent cells are growing in these conditions.

The yield of committed oligodendroglia remained relatively small. There may be many explanations for this; no attempt was made to induce differentiation in these cultures, and the cells may not have had time to differentiate to the O4 positive stage. Interestingly, cells detached from the adherent population with trypsin (the free floating aggregates were aspirated off without physical or enzymatic assistance first) and grown in similar conditions on poly-lysine, possessed more typical

oligodendrocyte progenitor morphology. However, while they stained with A2B5, they also stained positive for GFAP suggesting a "type 2" astrocyte identity (Figure 5-11). In fact, few A2B5 positive cells in this preparation were GFAP negative, irrespective of morphology. In contrast, cells derived from the suspended cells revealed a mixture of oligodendrocyte antigens but without GFAP co-staining (Figure 5-14).

Adult human stem cells can give rise to substantial numbers of oligodendrocyte lineage cells.

The demonstration of characteristic stem cell growth characteristics with appropriate mitogens, the expression of a mixture of stem and progenitor antigens and the differentiation of daughter cells into all three neural lineages all supported the hypothesis that stem cells could be grown from the adult human brain. However it remained unclear whether the oligodendrocyte yield from these cultures was sufficient to provide a viable source for therapeutic transplantation.

To address this required a further experiment where sphere progeny could be assessed quantitatively. It was decided to compare the current protocol with two alternatives suggested by contemporary reports that had used the aggregate culture approach. The first approach was to induce the formation of "oligospheres" similar to those reported form rat, mouse and canine brain (Avellana-Adalid et al. 1996;Vitry et al. 1999;Zhang et al. 1998a) using medium conditioned with the rat neuroblastoma line B104. The second was to use the conditions reported by a group, experienced at obtaining high oligodendroglial yield from other mammals (Zhang et al. 1998b;Zhang et al. 1998a), which had focussed on oligodendroglia formation from fetal human brain at 15-20 weeks gestation (Zhang et al. 2000). These cells had been expanded first as neural spheres using FGF2, EGF and Leukaemia Inhibitory Factor (LIF). The addition of LIF was reported as preserving their differentiation potential better than FGF2 and EGF alone, particularly with regards to oligodendroglia formation, although the final oligodendroglial yield remained below 5%.

Following dissociation along standard lines, cells were grown initially in EGF and FGF for three weeks to form free floating spheres. These cultures were then split equally into three different flasks in E&FGF, E&FGF with LIF or E&FGF with B104-conditioned medium for a further four weeks with regular growth factor replacement. Cells were then dissociated from the spheres using trypsin and EDTA and plated onto

poly-lysine coverslips in a differentiation medium containing low PDGF(1ng/ml) concentrations and 0.5% serum (Zhang et al. 2000) for a further week. Cells were then immunostained for markers of different neural lineages (O4, GFAP). (β_{III}tubulin staining on this occasion was unfortunately unreliable and has been omitted from the graph). Other putative markers of more immature oligodendroglia were assessed on parallel cultures at 2-4 days post dissociation and are included here (Figure 5-15). Observation prior to dissociation suggested some qualitative differences between groups. The cells grown in EGF & FGF alone had formed spheres that were between 100-150μm in diameter, while those in EGF, FGF & LIF were slightly smaller at between 50-100μm. Interestingly, those grown in B104-conditioned medium were very large (>300μm), although many of these larger aggregations were now irregular and appeared to be composed of several spheres that had adhered to each other. When the dissociated cells were identified there was surprisingly little variation in the percentage of each cell type found between the different conditions tested (Figure 5-15). However there was a notably high yield of oligodendrocyte lineage cells as identified by O4 antigen expression amongst cells from all conditions. This yield of around 60% contrasts starkly with that obtained from fetal human cells in similar circumstances (1-5% (Zhang et al. 2000)also (Palm et al. 2000)).

Discussion

The presence of neural stem cells in the adult brain is gaining credence. First hypothesised from developmental observations, neural stem cells have been identified, isolated and grown *in vitro* (Reynolds & Weiss 1996). Many of the properties of embryonic neural stem cells have been seen in cells dissociated from the mature mammalian brain, and these cells now appear to be present in the adult human (Akiyama et al. 2001a;Arsenijevic et al. 2001;Johansson et al. 1999;Kukekov et al. 1999). However, it should be noted that in none of these reports are all attributes of stem cells unequivocally demonstrated. One report does demonstrate clonality of many individual spheres by means of retroviral insertion analysis, and separately demonstrates multipotency by antigenic means, but these spheres failed to propagate beyond four passages – true assymmetric division would predict unlimited division. Others fail to demonstrate clonality with absolute security or use tissue sources that could contain transformed cells (Akiyama et al. 2001a).

The experiments described here broadly support the hypothesis that adult human brain contains stem cells. However, in view of the strict requirements for proving clonality, asymmetric division and true, functional multipotency it would be more accurate to conclude that *sphere-forming cells* are present in adult human brain, and that these cells proliferate to form multipotent spheres, staining widely for immature antigens, and demonstrating differentiation markers of all three neural lineages.

While this in itself is notable, it is not entirely new. However, the high proportion of O4 staining cells from these cultures is of great significance. Several reports suggest that oligodendroglia are a minority population from differentiating neurospheres. 1-5% is a common yield (Palm et al. 2000;Zhang et al. 2000). Other reports do not quantify the oligodendroglial yield. If adult human neural stem cells are to be a viable source of remyelinating cells for therapeutic purposes then prior commitment to the oligodendrocyte lineage may be an important first step (Smith & Blakemore 2000). Certainly, widespread differentiation of transplanted cells into unwanted neurons (which could be epileptogenic) or astrocytes (which could impede remyelinating cells (Fawcett & Asher 1999)) may be highly detrimental. Interestingly, when transplanted, putative neural stem cells (albeit tumour-derived), produced peripheral, not central myelin following transplantation. Tissue specific cues for lineage determination remain poorly understood. Peripheral myelin may be sufficient to provide short-term

functional benefit, but the requirement for axonal trophic support may mitigate against such a therapeutic strategy. The demonstration of a viable source of oligodendrocyte lineage cells expanded *ex vivo* from adult human brain is therefor encouraging.

Future work

Two important elements remain unaddressed. Firstly the phenomenal replicative potential remains to be fully realised. To some extent this may be amenable to relatively minor changes in technique. There were considerable difficulties in adequately dissociating spheres using purely mechanical means, despite trying fire-polished Pasteur pipettes, Gilson™ Pipettes and other means. In fact, high concentrations of both the chelating agent EDTA and trypsin were required to dissociate these cells into individual cells, a process that has been reported to be associated with a significant reduction in subsequent proliferation and sphere formation (Zhang et al. 2000). Furthermore, the importance of sphere-conditioned medium – that is undefined soluble factors secreted by proliferating spheres that encourage sphere formation and cell division in other cells with a similar potential, may have been underestimated (Zhang et al. 2000). It is clear also that there are cell-cell interactions within spheres that facilitate both proliferation and self renewal. The requirements for both a cell of stem potential, and a stem cell "niche" created by surrounding cells and the extracellular matrix are conjectured, but remain to be elucidated.

Secondly the progeny of these spheres need further assessment. As has been underlined in other chapters, we believe that identification by immunophenotype and morphology, while reproducible, convenient and generally reliable, is most robustly supported by functional analysis. In the oligodendrocyte lineage this means the sequential expression of differentiation antigens, contact with axons, ensheathment and maturation of the myelin sheath, and finally both restoration of saltatory conduction and axonal trophic support. These will require both co-culture experiments and transplantation studies to ascertain.

Transplantation studies require an adequate means of identifying transplanted from host cells. While various options are available at differing levels of technical complexity, the use of retroviral vectors allows both labelling, and, crucially, the most robust form of clonal analysis. While no *sine qua non* of a transplantation strategy,

rigorous confirmation of the presence of neural stem cells in the adult human brain would be of great scientific import.

Illustrations

Figure 5-1 Free floating, unpurified cells grown first in 10% FCS and then in EGF&FGF form aggregates on uncoated plastic. When transferred to a permissive substrate (PLL) they adhere and develop a network of interlocking processes. Some bipolar cells with the immunophenotype of oligodendrocyte progenitors are visible stained with A2B5(red), and more mature putative oligodendrocytes stain with GalC(green) (arrows). GFAP(blue) identifies a network of interconnecting astrocytic processes. Myelin debris also stains with oligodendrocyte antigens. Nuclei are identified by Hoechst(blue) (x100, Bar = 40μm).

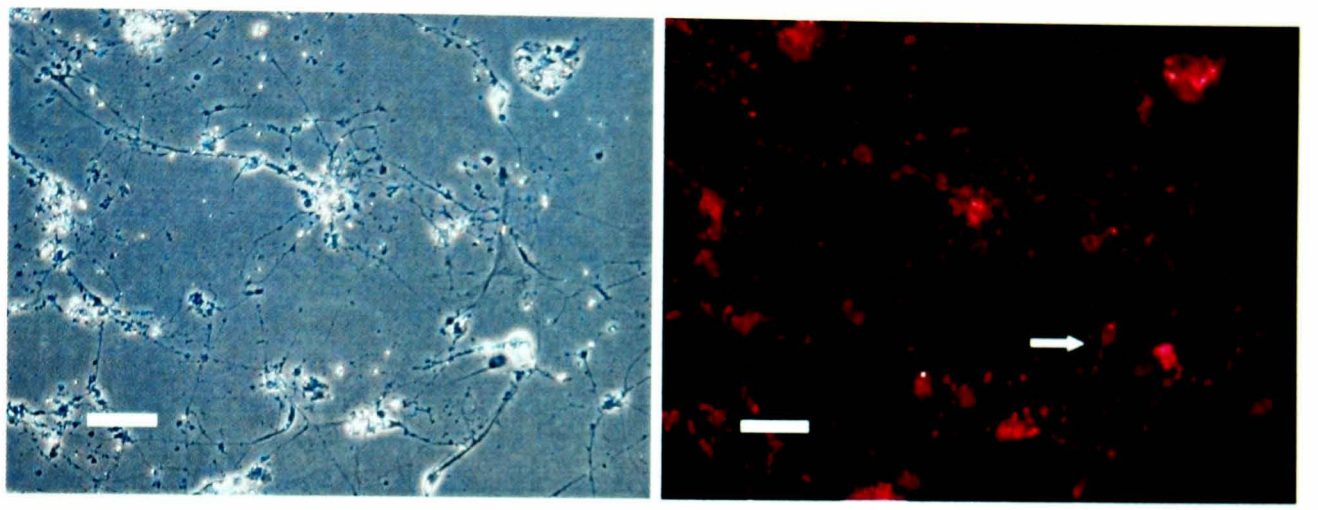


Figure 5-2 The same cell preparation as figure 1 under high power (x400). A processed astrocyte with bundles of cytoskeletal GFAP (blue), and a separate flattened cell with sheet like extensions staining strongly with the oligodendrocyte marker GalC (green) are visible. A2B5 staining was negative (not shown).

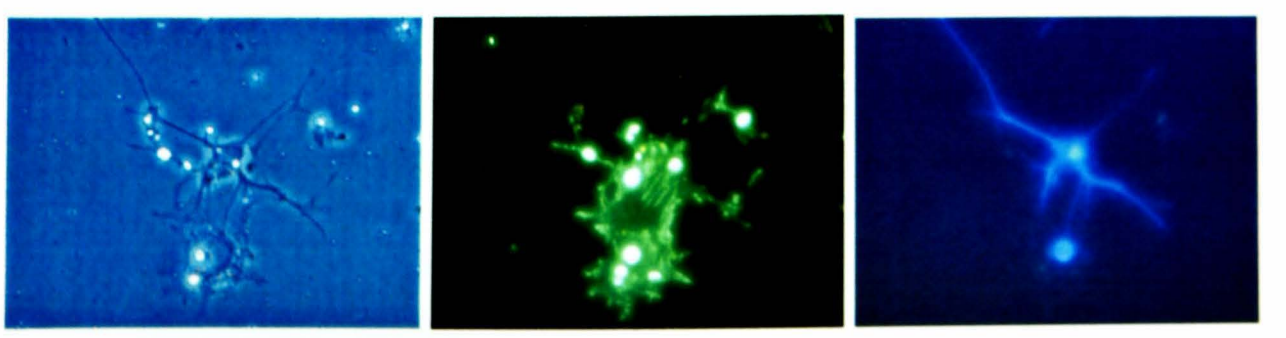


Figure 5-3 Occasional cells stained for both oligodendrocyte and astrocyte markers (GFAP-blue, GalC-green, A2B5-red x400).

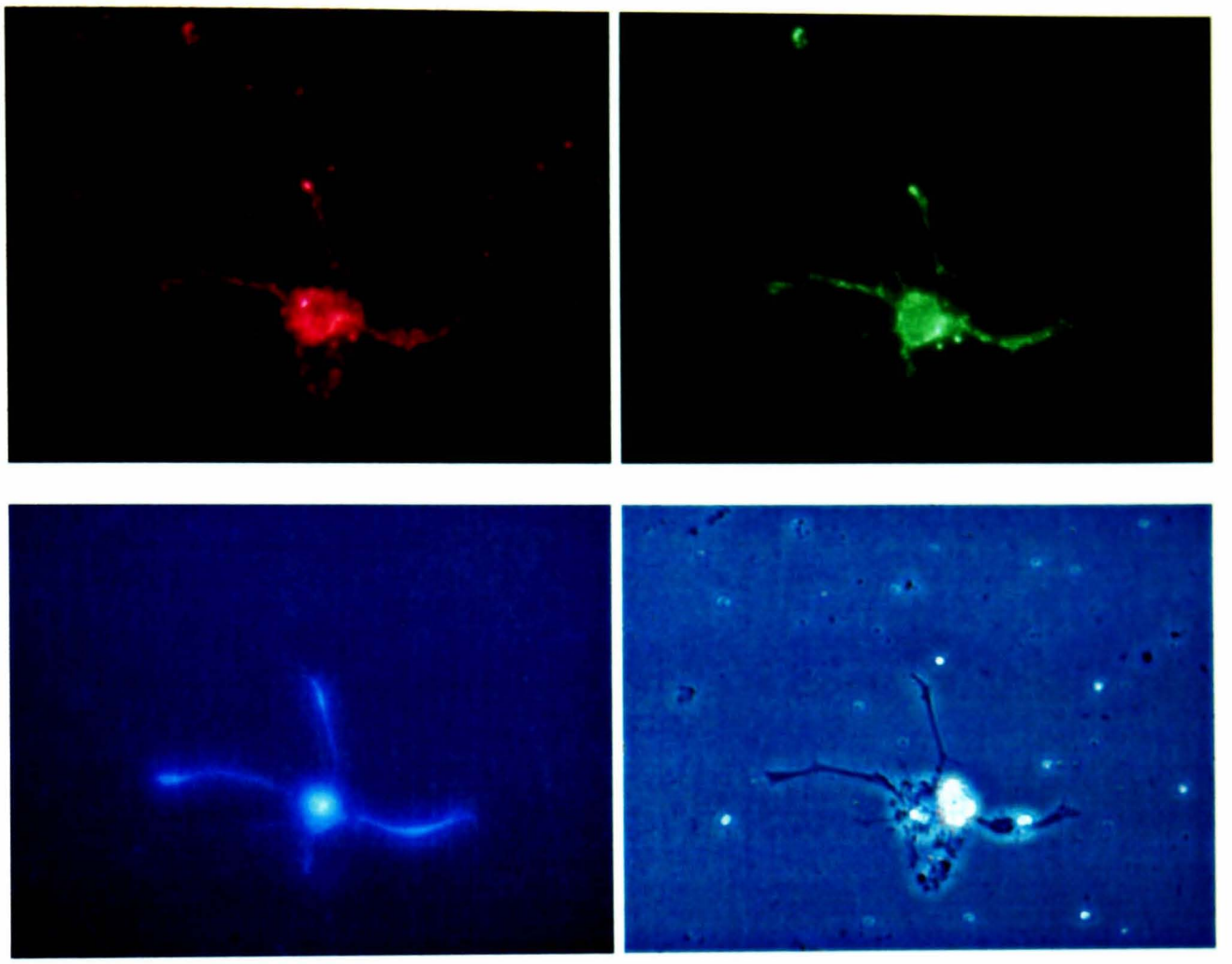


Figure 5-4 Some cells cultured *ab initio* in DMEM:F12 medium with FGF and EGF grow into spheres on uncoated tissue culture plastic, but other cells adhere, some of which appear to migrate out from the sphere (Phase contrast x100)

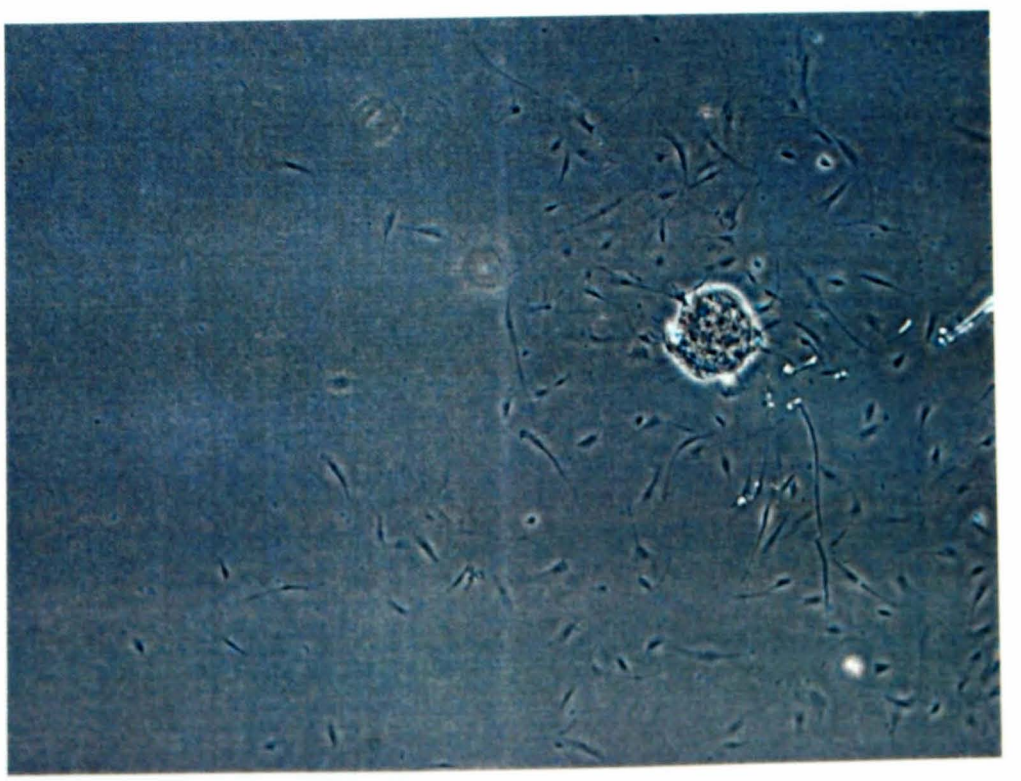


Figure 5-5 Cellular migration from E&FGF expanded spheres is enhanced by plating onto poly-lysine (phase contrast x100). Some of these cells stain weakly for Vimentin (red). Staining for neurofilament (NF200kD green & below x600) shows an atypical pattern not consistent with a cytoskeletal protein.

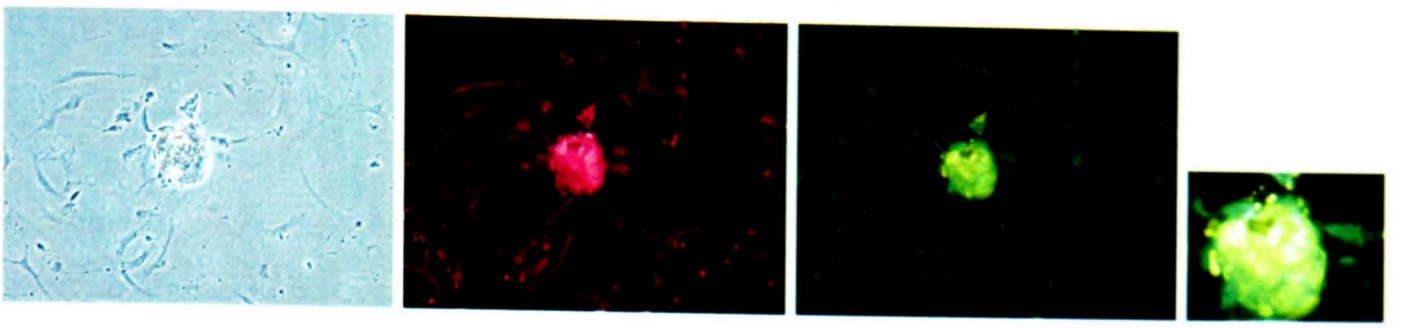


Figure 5-6 A separate sphere grown under the same conditions as Figure 5-5 (phase contrast x200) shows a mixture of normal and pyknotic nuclei labelled with Hoechst, and 2 emerging astrocytes labelled with GFAP (blue). There is evidence of A2B5 (red) and GFAP (green) staining of the sphere, but not of the adherent cell population.

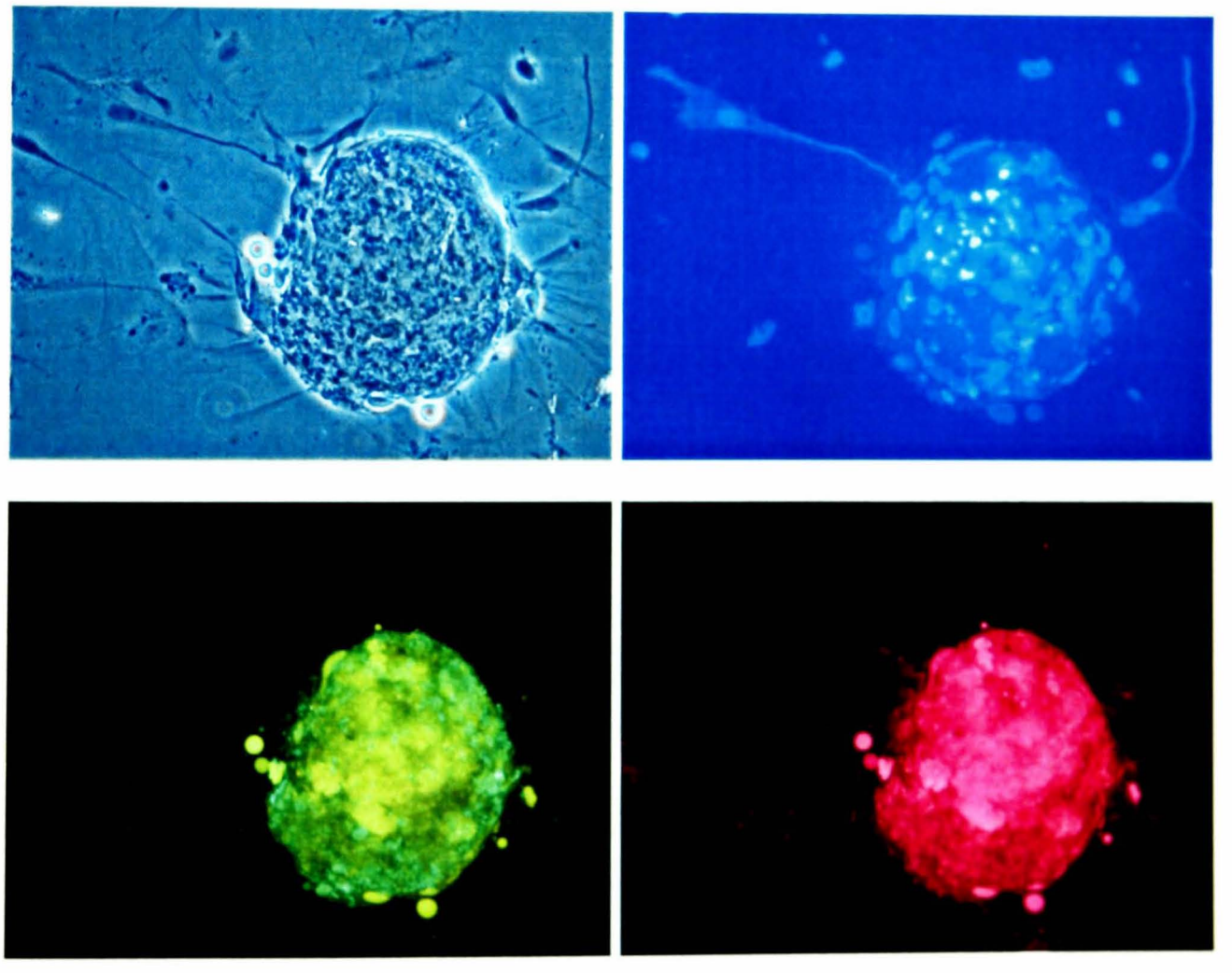


Figure 5-7 S_{100} staining was also evident in a subgroup of adherent cells

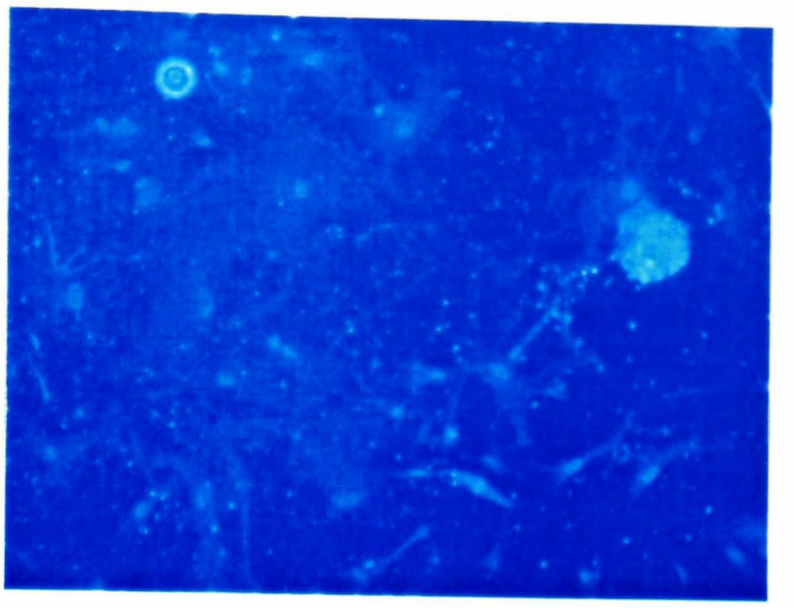


Figure 5-8 The Effect of Different Growth Conditions on the oligodendrocyte and astrocyte lineages (Adh = adherent conditions, Float = free-floating, Hi.Gluc = 6% glucose, Lo.Gluc = 3% glucose) [Analysis of 12 parallel coverslips, included here for illustrative purposes only – see text]

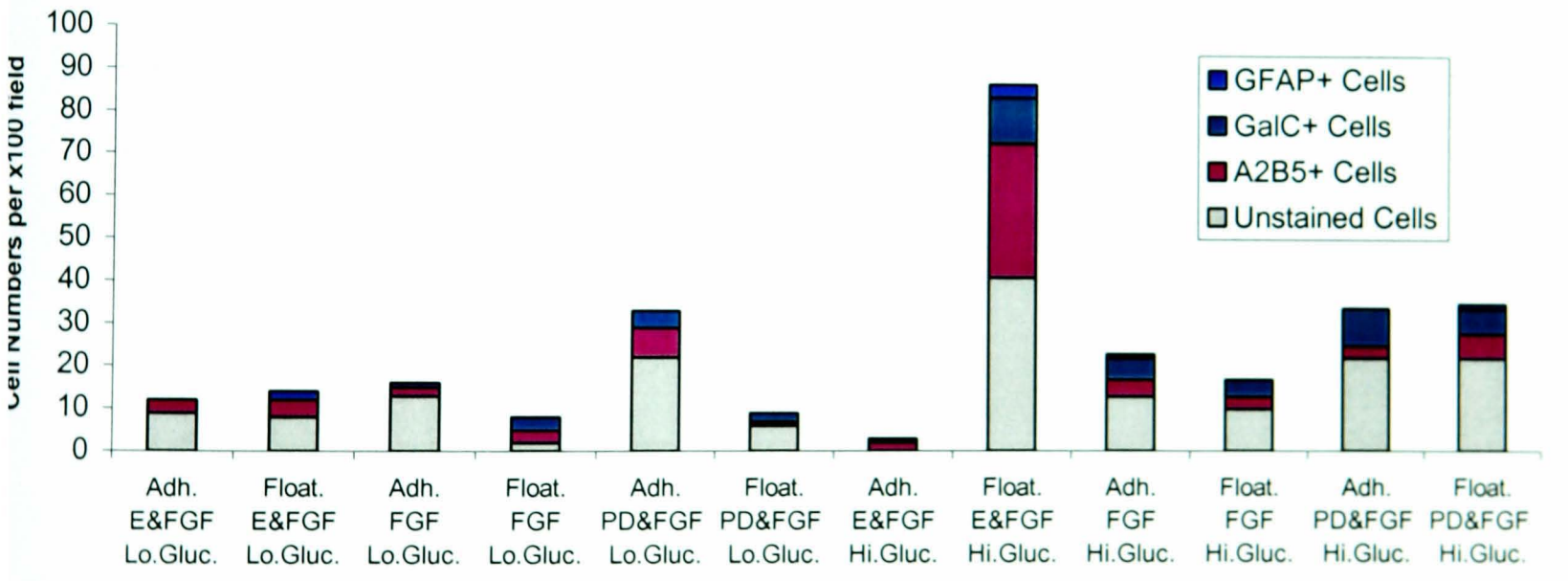


Figure 5-9 Spheres grown in EGF and FGF (top left and right; scale bar = 50μm) plate down spontaneously if growth factors are not regularly replenished, and manifest various morphologies (bottom left and right; scale bar = 20μm).

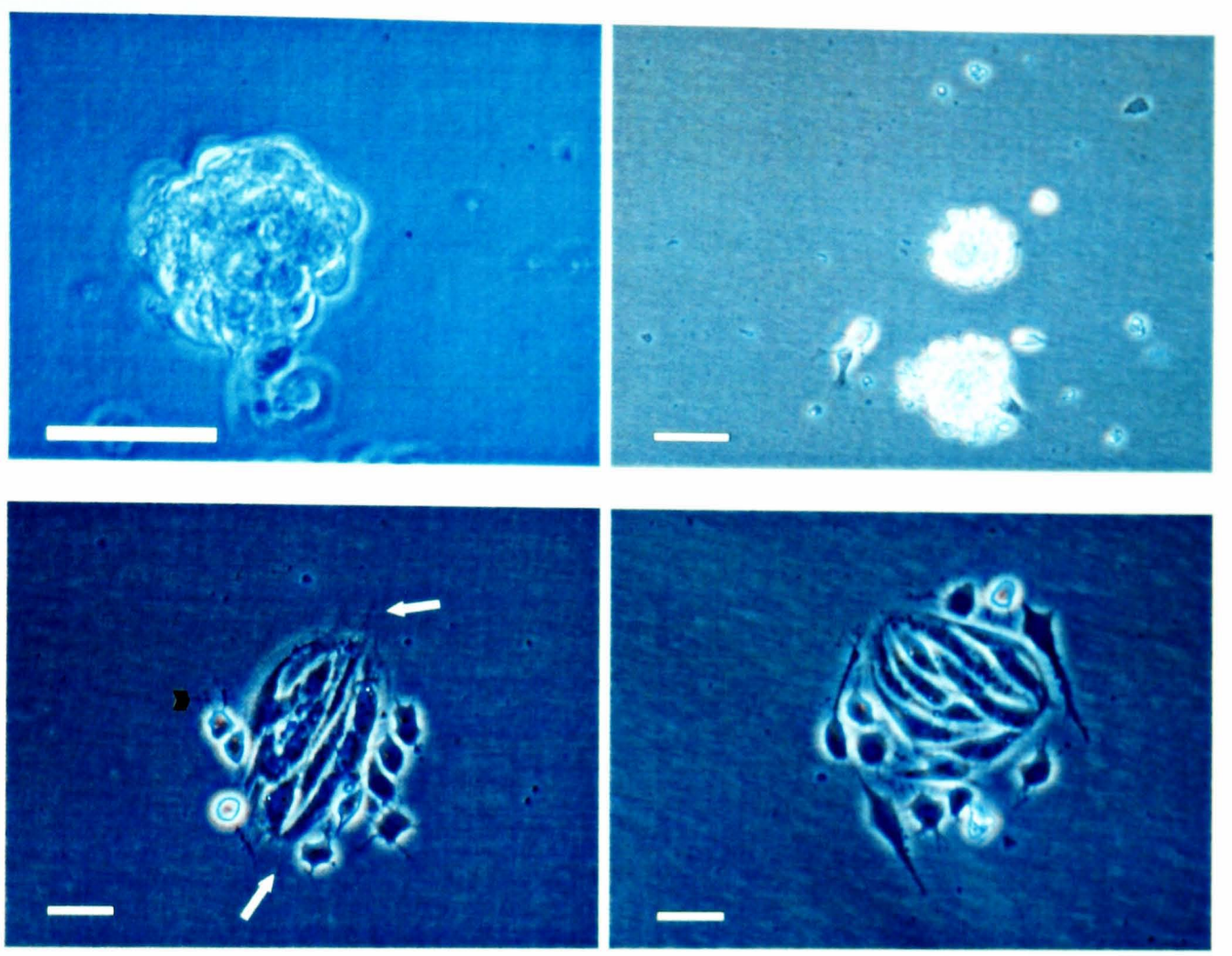


Figure 5-10 Free-floating aggregates adhere to poly-lysine substrates and express markers of early progenitors; Nestin (Red) and PSA-NCAM (green) (Scale Bar = 20μm)

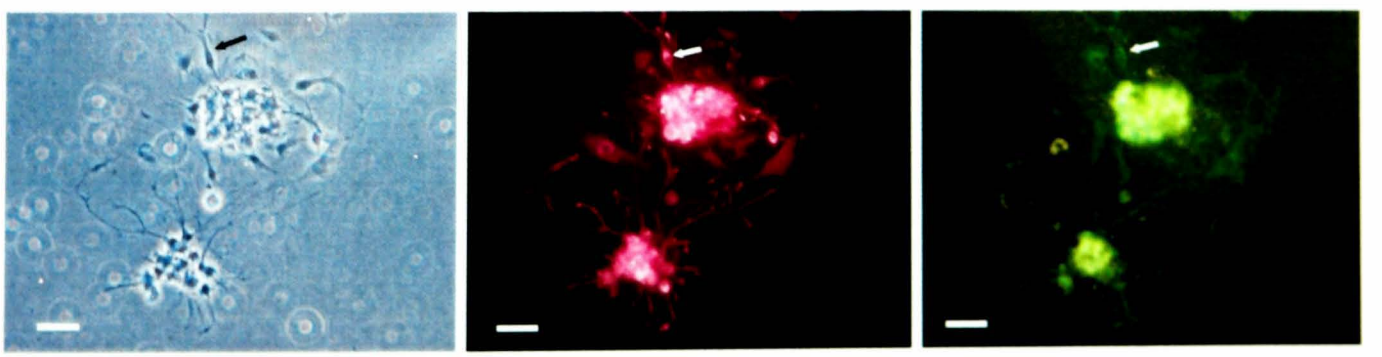


Figure 5-11 After a week on poly-lysine, markers of neurons (β_{III}tubulin – green) and astrocytes (GFAP – blue) are expressed by different subpopulations. (Scale Bar = 20μm)

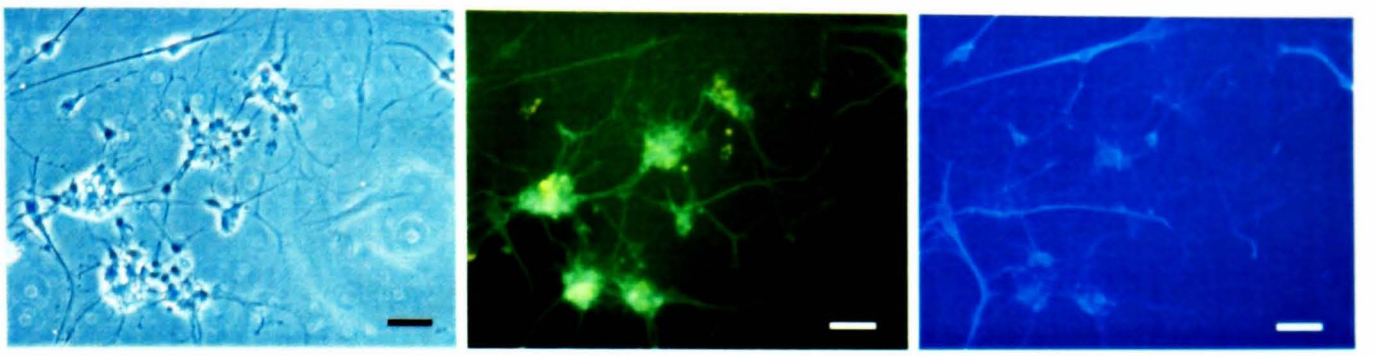


Figure 5-12 Parallel cultures demonstrate that oligodendrocyte lineage cells identified by the O4 antibody (red) are also present in these adhering aggregates (Scale bar = 20 μm)

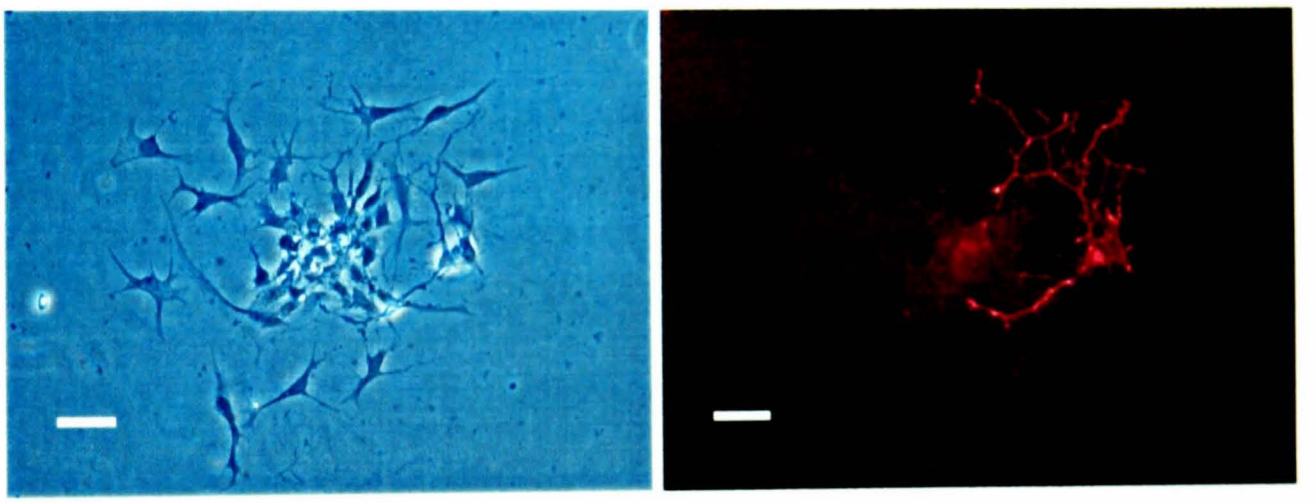


Figure 5-13 Bipolar cells, which appear to originate from a loosely adherent population growing alongside the aggregate cultures, can be dislodged with trypsin, re-adhere to poly-lysine and stain variously for A2B5 (red), GalC (green) and GFAP (blue). (Scale Bar = 20μm).

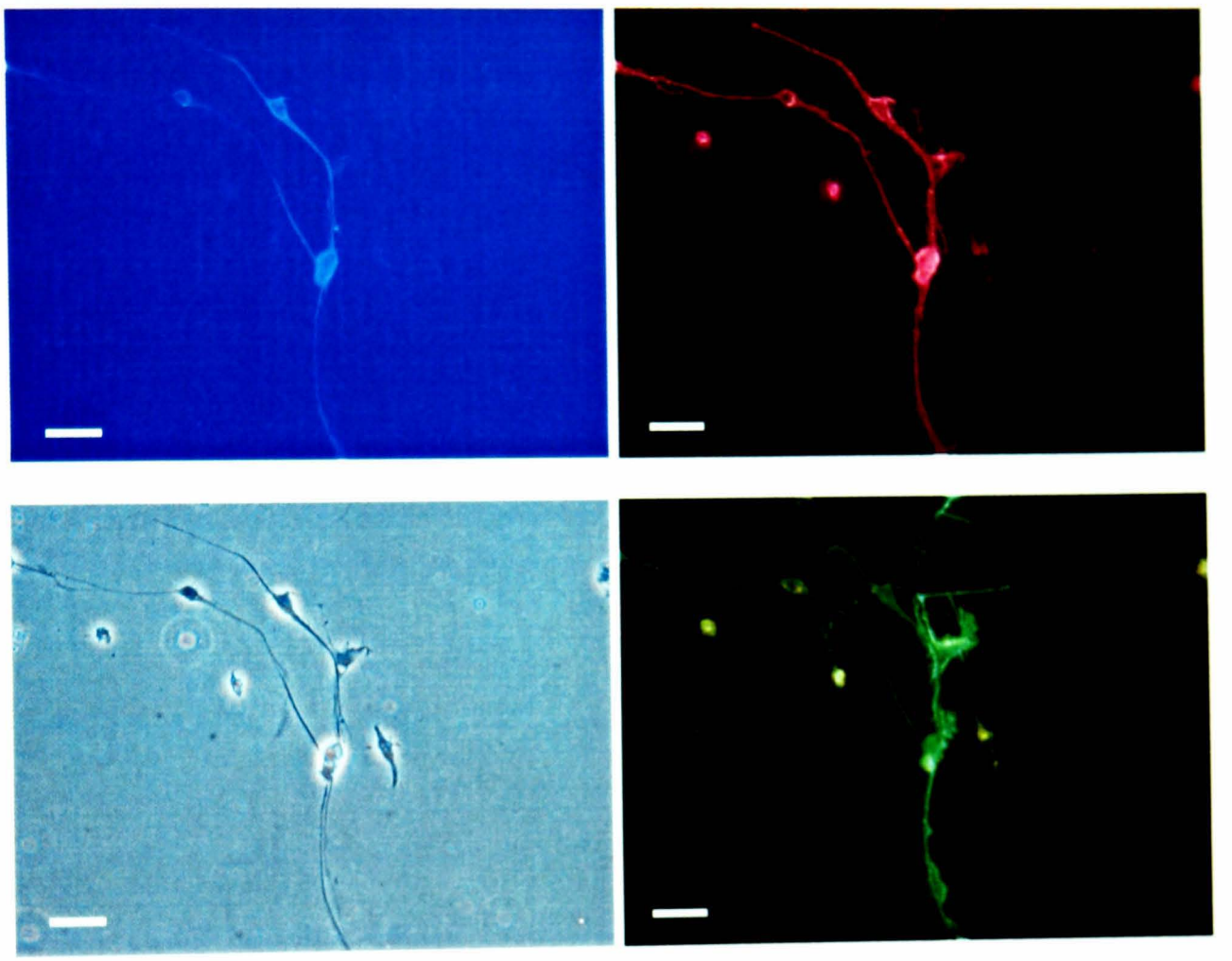


Figure 5-14 After aggregate cultures are transferred to adhesive substrates, some cells also express putative oligodendrocyte markers; A2B5 (green) and AN2 (a homologue of NG2) (red) but not GFAP (blue). (Scale Bar = 20μm)

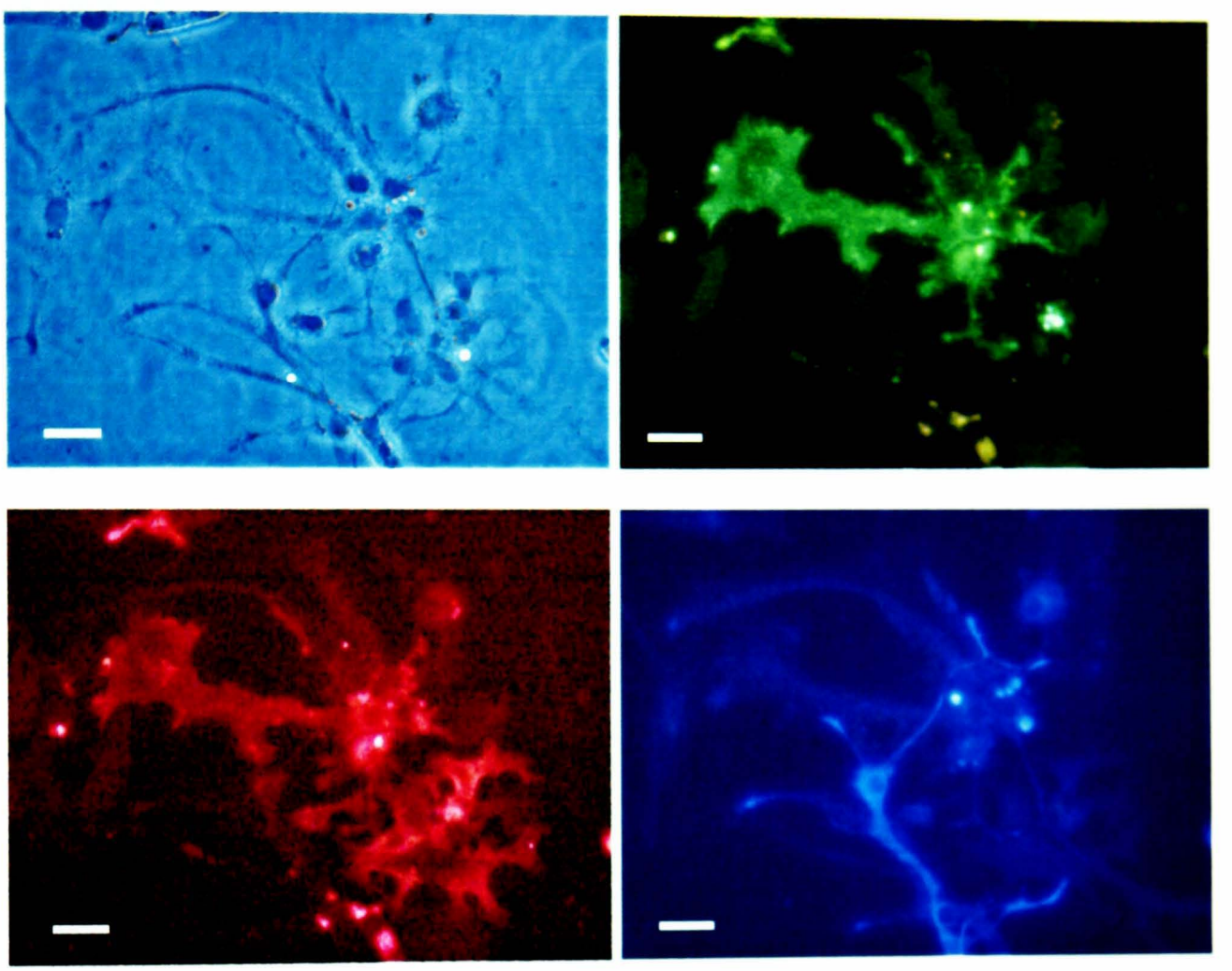
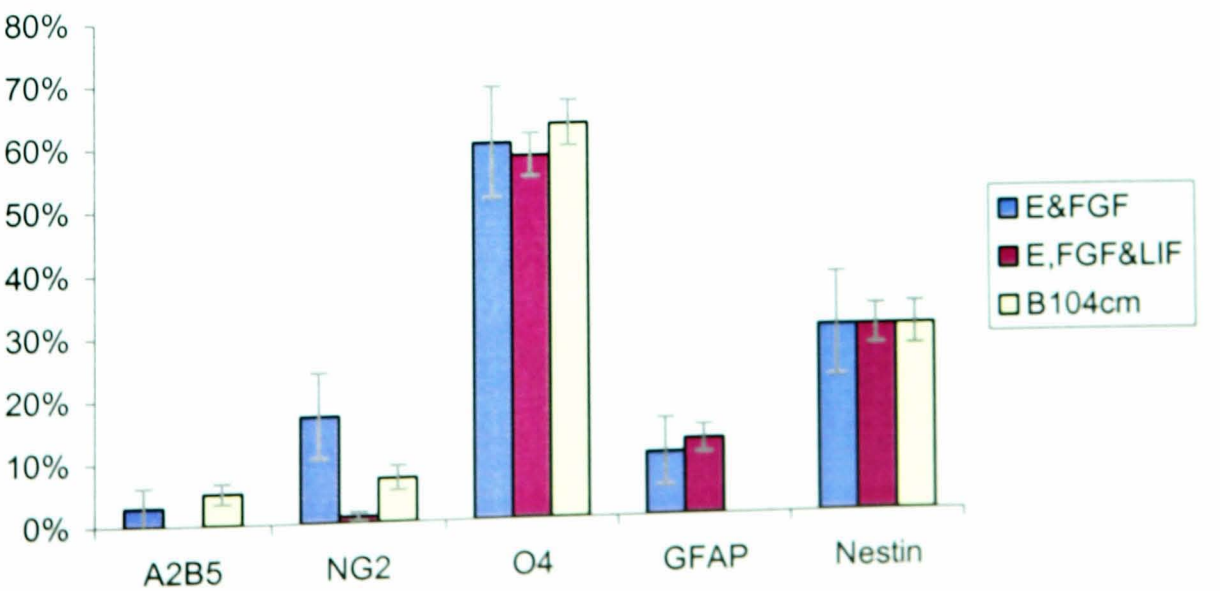


Figure 5-15 Different growth factors make little apparent difference to the immunophenotype of the dissociated cell population. [Parallel analyses, single human sample, 2 coverslips per culture condition, 10 random x200 power fields per coverslip, per immunolabel]



Chapter 6. Oligodendrocyte Progenitors (CG-4) Are Resistant to the Effects of Immune-Modifying Agents *In Vitro*

Introduction

An example of one of many questions that requires a source of human oligodendrocyte progenitors to answer definitively, is whether anti-inflammatory drugs used to suppress disease activity in multiple sclerosis may impair the myelin repair process. The absence of such a source means alternative models have to be used, in this case, the rodent oligodendrocyte progenitor cell line CG4. These studies exemplify the numbers of cells required to answer these crucial questions of cell biology, and the way in which such important questions can be addressed.

The pathology of Multiple Sclerosis (MS) is dominated by oligodendrocyte damage and myelin loss. Intrinsic repair in the form of remyelination occurs in MS (Perier & Gregoire 1965), but is limited and ultimately fails to prevent the progression of disability. Quiescent oligodendrocyte progenitor cells residing in normal adult brain divide and migrate into demyelinated lesions, and are thought to be largely responsible for new myelin formation (Blakemore & Keirstead 1999;Carroll & Jennings 1994;Scolding & Franklin 1997). In MS, this is seen as areas with unusually thin myelin sheaths predominantly distributed towards the edge of lesions. Experimental remyelination suggests that this repair restores robust saltatory axonal conduction with demonstrable functional benefits (Jeffery et al. 1999;Utzschneider et al. 1994). Furthermore it may protect axons from subsequent degeneration (Halfpenny et al. 2002;Kornek et al. 2000;Scolding & Franklin 1998). Consequently therapeutic remyelination strategies are being actively pursued with considerable enthusiasm and optimism; indeed early clinical studies implanting myelinating cells into patients are already underway (http://www.myelin.org/schwannupdate.htm).

Immune mediated myelin and oligodendrocyte destruction appears to be predominant in MS, and many immunomodulatory drugs have been assessed in clinical practice. Of these, corticosteroids and interferon-beta (IFN-β), and to a lesser extent azathioprine, are widely used and are efficacious in relapsing-remitting disease (Milligan et al. 1987;Sellebjerg et al. 1998;The IFNB MS Study Group 1993;The OWIMS study group 1999;Yudkin et al. 1991). Despite the increasingly recognised importance of remyelination in MS, there is little understanding of whether or how these drugs may

affect myelin repair, either spontaneous or putative therapeutic approaches. Since such reparative strategies are already now being implemented, it is timely to explore the possibility that these drugs might significantly affect remyelinating cell function.
It is well established that two basic biological characteristics; migratory capacity and mitotic potential, are important determinants of myelinating cell efficacy (Blakemore & Keirstead 1999;Franklin & Blakemore 1997). There is limited evidence that steroids inhibit oligodendrocyte proliferation (Alonso 2000); type I interferons (α&β) have an anti-proliferative effect on some cell types (Lin et al. 1986;Moore et al. 1984), and azathioprine is a more potent anti-mitotic agent: these effects could have a significant impact on the behaviour of oligodendrocyte progenitors in acute demyelinating lesions.
Here we use an *in vitro* approach to assess firstly potential direct cytotoxic effects of these drugs, and secondly to explore both proliferation and migration of oligodendrocyte progenitors under the influence of three immunomodulatory agents used widely in multiple sclerosis: corticosteroids, IFN-β and azathioprine.

Materials and Method

Determining Drug Concentrations

Pharmacological studies vary in their pertinence to drug regimes used in MS, making direct extrapolation of drug concentrations for use *in vitro* problematic. Thus wide ranges of concentrations were used aiming to investigate drug concentrations approximately 2 orders of magnitude above and below those thought to be clinically relevant, as guided by available *in vivo* studies. Peak levels of methylprednisolone reach 0.5μg/ml in the cerebrospinal fluid of MS patients after a 1.5g infusion (Defer et al. 1995), and levels of IFN-β peaked at approximately 4 IU/ml 4 hours after a single 60μg injection of IFN β_{1a} (Munafo et al. 1998). Levels of azathioprine have to be extrapolated from non-MS patients and studies have shown significant differences in steady state levels between extensive metabolisers, whose steady state remains below 27ng/ml irrespective of oral dose, and a small proportion (~30%) of poor metabolisers whose levels may reach 600ng/ml (el Yazigi & Wahab 1993).Although some of azathioprine's biological activity resides in the parent compound (el Yazigi & Wahab 1993), its principle metabolite 6-mercaptopurine may be responsible for much of its

clinical effect, and levels of this compound appear to be more predictable, with levels of 15ng/ml being recorded (Weller et al. 1995).

Anti-Inflammatory Drugs

Stock solutions were made by dissolving reagents (all obtained from Sigma, Poole UK unless otherwise specified) in methanol (methylprednisolone) or 1M NaOH (azathioprine & 6-mercaptopurine). IFN-β was available in solution. All further dilutions were made into Sato medium containing BSA and pH corrected where required. Vehicle controls were used where appropriate. In particular, the experimental protocol required the presence of a significant quantity of met hanol (final concentration 1%) in the highest concentrations of methylprednisolone.

CG-4 Cell Culture

CG4 cells were grown as previously described. Briefly, the cells were grown on poly-D-lysine (PDL) coated flasks in Sato medium (containing DMEM, 2mM L-glutamine, 5μg/ml insulin, 50μg/ml holo-transferrin, 100 μg/ml bovine serum albumin, 16 μg/ml putrescine, 60 ng/ml progesterone, 5 ng/ml sodium selenite and 400 ng/ml each of thyroxine (T_4) and tri-iodothyronine (T_3)), supplemented with 30% B104 conditioned medium. Cells were passaged 1:3 every 3-4 days. For the migration assay, cells were harvested from flasks with trypsin when about 80% confluent.

MTT Assay

CG4 cells in the logarithmic phase of growth were plated uniformly into PDL-coated 96 well plates (Nunc) and incubated for 24 hours in FGF2 (10ng/ml) alone or FGF2 and $PDGF_{AA}$ (10ng/ml) (Peprotech). Serial dilutions of test reagents were added across the plate, with both positive and negative controls in parallel, and the incubation was continued for a further 90 minutes (to assess cytotoxicity) or 24 hours (to assess proliferation) (Mosmann 1983). Cytosine arabinoside was used as one of these controls for its toxic effect on dividing cells. MTT was added to a final concentration of 1mg/ml for 1 hour at 37°C. The supernatant was then aspirated off, the formazan product dissolved in isopropanol and the reading measured with a Multiskan Ascent spectrophotometer. All assays were repeated with different passage numbers and using

different layouts to avoid systematic variations across the plates. Results shown are of a representative experiment.

BrDU Incorporation Assay

CG4 cells were plated on PDL-coated 13mm glass coverslips in multi-well plates and allowed to adhere overnight. Each well was then flooded with Sato medium supplemented with FGF2 and $PDGF_{AA}$ and different test reagents. Incubation was then continued for a further 24 hours. Bromodeoxyuridine was added to each well for the last four hours of this, to a final concentration of 10μM, before fixing in 4% paraformaldehyde for 5 minutes. The cells were then permeabilised for 10 minutes in ice-cold methanol and DNA was partially denatured in 2N HCl for 30 minutes at 37°C. Following a neutralisation step in borate buffer pH 8.5 they were labelled with anti-BrDU antibodies (Roche), washed and revealed with anti mouse IgG_1-FITC secondary antibodies. BrDU positive cells and total cell numbers were identified in at least 10 high power visual fields taken at random using an Olympus IX70 inverted immunofluorescence microscope with a x40 objective lens, counting a minimum of 200 cells per coverslip.

Agarose Drop Migration Assay

CG-4 cells growing exponentially were harvested with trypsin, washed through Sato medium, suspended in 40μl of Sato with 10% fetal bovine serum and kept at 37°C in a water-bath. To this was added 20μl 1% agarose in phosphate buffered saline and mixed thoroughly. The agarose drops were formed by seeding 1.5μl of this solution onto PDL coated multi-well plates and allowing the agarose to set for 10 minutes at 4°C. Sato medium, together with the test reagents were added gently so as to avoid disturbing the droplet. B104 conditioned medium was omitted from the migration experiments, but FGF2 was added to the test wells and controls to prevent the CG-4 cells differentiating. Limited migration occurred in the absence of growth factors - but this was terminated by the acquisition of a more ramified, immotile morphology (**Fig. 3**). Migration was measured at 40 hours using an Olympus IX-10 inverted microscope with a x10 objective lens and a graticule in the eyepiece. Measurements were taken by recording the distance travelled by the furthest cell from the edge of the agarose droplet in each of four quadrants (0, 90, 180 and 270°). Occasionally, agarose drops

became dislodged from the substrate, but this was easily detected and these results were discounted from the final analysis. Because proliferation of cells in the halo can contribute to the measured migration distance, certain experiments used aphidicolin (20 μg/ml), a mitotic inhibitor, to block this effect (Frost et al. 2000).

Statistical Analysis

All experiments were repeated at least three times on separate occassions with consistent results. Migration distances from the droplets were normally distributed within each experimental group. To correct for systematic differences between experiments, migration distances were converted to a migration index. This was calculated by dividing the distance migrated in the test wells by the mean distance migrated in the control wells and expressing this as a percentage.
All results were processed using Microsoft Excel and SPSS version 10 software. Migration and MTT results were analysed using a one-way ANOVA and where significant at the 5% level, subgroup analysis was performed with Dunnett's t-test.

Results

Cytotoxicity

We looked first to see whether any of the drugs had a cytotoxic effect on CG4 cells. Methylthiazoletetrazolium (MTT) is reduced to a formazan dye by viable cells but not by dead cells, and can be used as an assay for cellular injury (Mosmann 1983). CG4 cells incubated for 90 minutes in the presence of pharmacological levels of these reagents showed no fall in MTT value compared with untreated controls. However very high dose methylprednisolone (100μg/ml) did show a fall in viability (33%), although this did not reach statistical significance ($p=0.066$).

Proliferation

We used both the MTT assay and BrDU incorporation to assess proliferative activity. When incubated for 24 hours in the presence of the growth factors FGF and PDGF, CG4 cells proliferate, an effect that can be measured by a corresponding rise in their ability to reduce MTT (Mosmann 1983). We looked to see whether any of these drugs might affect this proliferative response (Figure 6-1). Again, only supra-pharmacological doses of methylprednisolone impeded this response ($p<0.001$), leading to a 71% reduction in MTT signal (and by inference, cell numbers). This effect exceeded that of cytosine arabinoside (AraC) ($p=0.044$), a selective inhibitor of DNA synthesis used as a control, suggesting that non-dividing cells might also be damaged by this dose.

Bromodeoxyuridine (BrDU) is incorporated into cellular DNA during the S-phase of the cell cycle, thus labelling actively dividing cells. Measuring the proportion of cells incorporating BrDU in a defined period enables a more direct measure of cellular proliferation (Figure 6-2). This confirmed earlier findings that these drugs have no direct effect at more pharmacologically pertinent doses. No effect on proliferation was observed with any of the drugs and doses examined.

Migration

Migration was assessed using the agarose drop assay as modified by Frost (Frost et al. 2000). FGF2 was used to maintain CG4 cells in an undifferentiated phenotype for the

duration of the migration assay, without which cells started to migrate normally, but quickly became multipolar and sessile (Figure 6-3).

Neither preliminary dose–ranging experiments, nor more statistically powerful studies disclosed any significant effects on migration mediated by the drugs tested (Figure 6-4a-d). Several different time periods were also investigated but no consistent effects were observed. A modest reduction in migration was seen with methylprednisolone at the highest dose investigated - 10μg/ml (Figure 6-4a), but on this occasion a similar reduction was seen with an equivalent methanol concentration used as the vehicle control. Neither of these approached statistical significance.

In the first series of experiments $PDGF_{AA}$ was used as a positive control. This consistently and significantly ($p<0.001$) promoted migration (Figure 6-4a-d). FGF2 and $PDGF_{AA}$ are established mitogens for CG4 (Louis et al. 1992), and the agarose drop assay may be confounded by alterations in cellular proliferation. The addition of aphidicolin, an inhibitor of mitosis, reduced the number of cells seen in the halo, and had a modest effect on the maximum migration distance as measured. However this protocol did not reveal any hitherto unobserved drug effects, while $PDGF_{AA}$ continued to stimulate migration in this situation. This supports previous reports that $PDGF_{AA}$ stimulates progenitor migration in rodent OPCs independently of proliferation) (Decker et al. 2000;Noble et al. 1988;Simpson & Armstrong 1999).

Remyelination is thought to occur under the influence of astrocyte-derived growth factors, such as $PDGF_{AA}$. We investigated the possibility that these agents might act by inhibiting PDGF-stimulated migration. We therefore repeated the experiments using both FGF2 and $PDGF_{AA}$ in all wells, together with the test reagents. Again, consistent augmented migration was seen, but no drug-induced effects were observed (Figure 6-5).

Discussion

The reasons for remyelination failure in MS remain to be fully defined. Progenitor numbers in lesions may become depleted, the milieu within lesions may become unfavourable over time (Fawcett & Asher 1999) and/or persistently demyelinated axons may lose their potential to be remyelinated (Charles et al. 2002). Limitations of proliferative or migratory capacity are implicated either as key causes of, or contributors to this failure (Franklin 2002) .

IFN-β, high-dose corticosteroids and, to a lesser extent, azathioprine have a proven, if limited efficacy in suppressing immune activity and relieving clinical symptoms in relapsing-remitting MS (The IFNB MS Study Group 1993;Yudkin et al. 1991). However, their impact on progressive disability has been disappointing (Kappos et al. 2001;The IFNB MS Study Group 1993;The PRISMS Study Group 2001). This may reflect important differences in the mechanisms underlying acute and chronic disease. Immune processes are relatively quiescent in chronic progressive disease, but it remains surprising that the beneficial effect of reducing immune mediated damage is not reflected in greater long-term functional gains. This raises the possibility that treatment may have other less beneficial effects. Remyelination contributes to recovery from acute relapse and this process starts during the acute inflammatory phase (Smith & McDonald 1999), thus even steroids, given solely during the acute attack, may have the opportunity to interfere with remyelinating oligodendroglia.

The evidence concerning the effects of immunomodulatory agents on remyelination is at present conflicting. *In vivo* studies have explored spontaneous remyelination following toxic demyelination in rodents. In mice methylprednisolone (45mg/kg) accelerated remyelination (Pavelko et al. 1998) while in rats dexamethasone (3mg/kg/day) reversibly delayed remyelination. Azathioprine (15mg/kg/day) accelerated remyelination and increased myelin thickness (Herndon 1987). However these drugs produced profound variations both in the degree of demyelination and of the inflammatory infiltrate (Herndon 1987), making interpretation of the mechanisms for these effects problematic. Others have shown some promotion of remyelination in a virus induced demyelinating mouse model by type I interferons (Njenga et al. 2000). In more recent studies a clear inhibitory effect of immunosuppressive drugs on remyelination was demonstrated (Smith & Franklin 2001)

We attempted to circumvent these problems by taking a reductionist approach. Having shown no direct cytotoxicity, we looked specifically at direct drug effects on two biological properties of oligodendrocyte progenitors, proliferation and migration. Experimental work has repeatedly identified these as necessary and important for efficient remyelination (Blakemore et al. 2000;Crang et al. 1998), and stimulating both these properties, by the infusion of PDGF, yields a corresponding increase in myelinating cell efficacy (Allamargot et al. 2001).

Of these agents, only the effects of corticosteroids on basic oligodendrocyte biology have been investigated in any detail, and their role in proliferation and differentiation is complex. Exogenous corticosteroids appear to delay the onset of developmental myelination *in vivo* but normal myelin formation follows its withdrawal (Huang et al. 2001). Other studies have demonstrated a reduction in BrDU uptake by parenchymal NG2+ve cells (putative oligodendrocyte progenitors) in adult rats treated with corticosterone. These cells did not stain for the glucocorticoid receptor, prompting the authors to propose that indirect mechanisms might be involved (Alonso 2000), although *in vitro* all stages of the oligodendrocyte lineage from progenitors to oligodendrocytes appear to express mRNA for the glucocorticoid receptor constituitively (Chan et al. 1998). Barres et al. implicated corticosteroids, along with thyroid hormone and retinoic acid, as alternatives necessary for the effecter mechanism of a cell clock, although the evidence is slightly in favour of thyroid hormone playing this role in physiological conditions (Barres et al. 1994). Later in cellular maturation glucocorticoids appear to play a role in the initiation of myelination (Chan et al. 1998) and promote myelin formation by controlling the translation of myelin basic protein and proteolipid protein, as well as stimulating myelin lipid production (Kumar et al. 1989).

Our results help to clarify this picture. We did not see a reduction in proliferation *in vitro* to mirror that reported by Alonso (Alonso 2000) except at doses that are unlikely to be clinically relevant. In addition, both azathioprine and IFN-β had no demonstrable effect on proliferation, and no agent directly affected migration. The assays used were sensitive to the effects of the control agents, so the absence of any positive or negative effects on the drugs tested thus appears more robust.

We conclude that direct inhibition of remyelinating cells by these drugs is unlikely. This would suggest that the effects on remyelination previously observed *in vivo* (Herndon 1987;Njenga et al. 2000;Pavelko et al. 1998;Triarhou & Herndon 1986)

were caused indirectly. There is some evidence to support this. The role of the inflammatory response in promoting remyelination has been explored in animal models of de- and remyelination. These have suggested that macrophages can have important trophic effects (Kotter et al. 2001), perhaps through secreted inflammatory mediators such as IL1β (Mason et al. 2001) or TNFα (Arnett et al. 2001), previously thought to fulfil a more malign role in the pathogenesis of MS and oligodendrocyte biology (Andrews et al. 1998;Cammer 2000;Cammer & Zhang 1999). This may help to explain why potent immunosuppression appears to be associated with a reduction of remyelination in some of these models (Smith & Franklin 2001;Triarhou & Herndon 1986). The possibility of indirect effects on migration also exists; migration and adhesion of OPCs on astrocyte monolayers can be modulated by cytokines that act primarily on the astrocytes (Fok-Seang et al. 1995).

Questions of basic oligodendrocyte biology assume new relevance if oligodendrocyte progenitor transplantation for MS becomes a clinical reality. Clinical experiments wherein autologous remyelinating glia are implanted into brain lesions in patients with MS have commenced and more are expected. Notwithstanding the limitations of *in vitro* studies, our findings imply that any future relapsing disease activity in these patients can be treated in conventional ways with a little more confidence that the drugs used will not compromise the integrity or function of implanted oligodendrocytes.

Illustrations

Figure 6-1 Anti-inflammatory drug dose-response curves for proliferation.

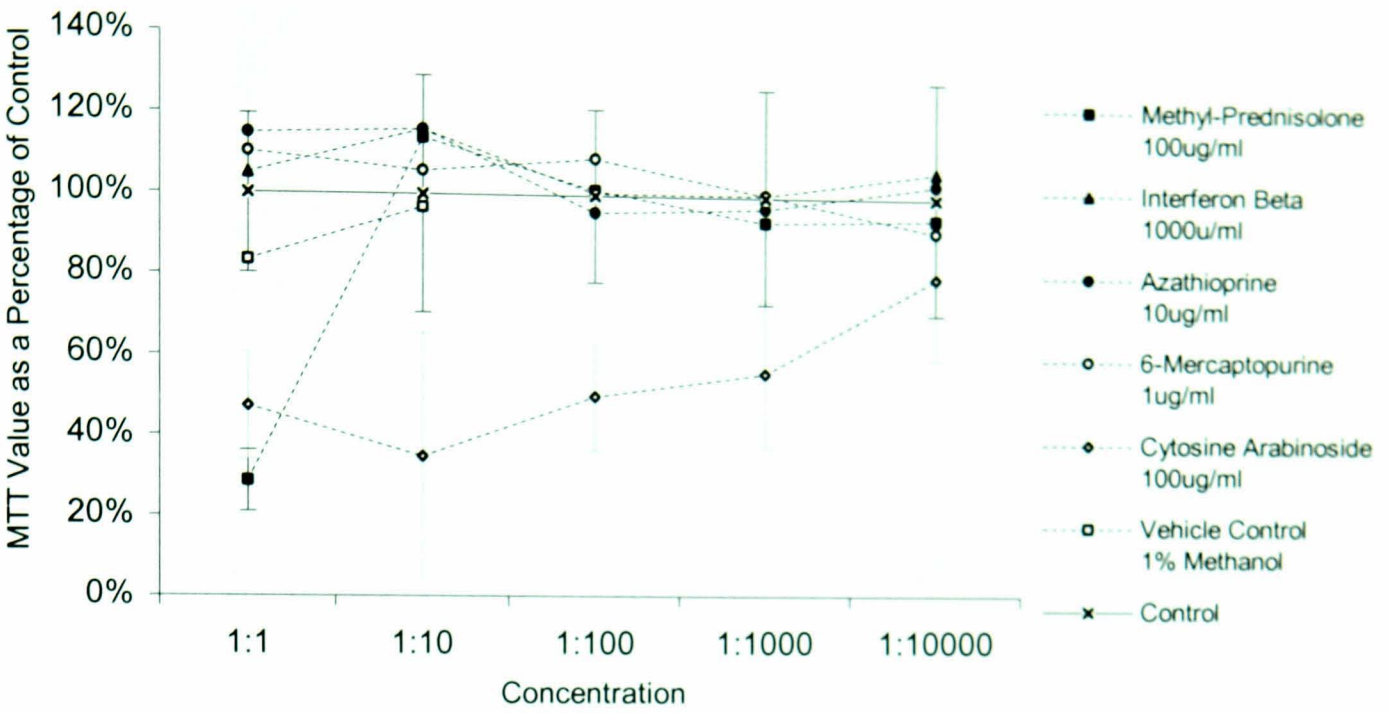


Figure 6-2 Pharmacological doses of anti-inflammatory drugs show no measurable effect on cell division.

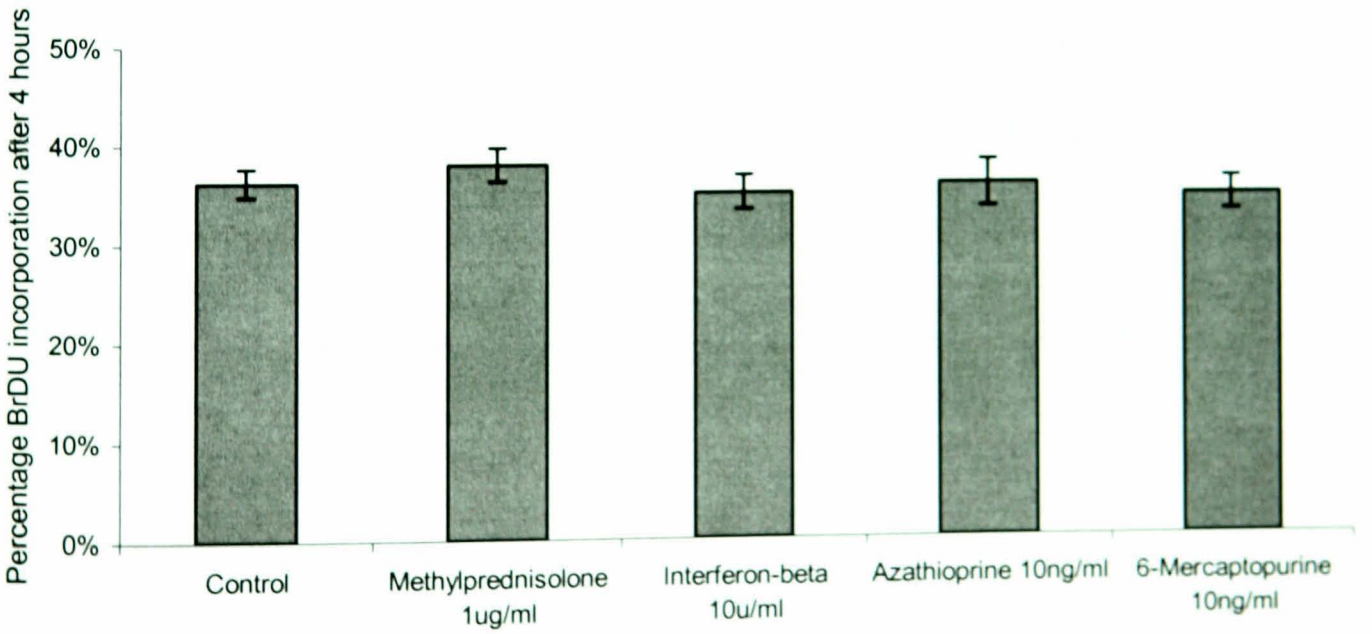


Figure 6-3 CG4 cells held in suspension by an agarose droplet contact the substrate and migrate radially. This distance can be easily measured (double arrow) However in the absence of growth factors (top), migration ceases as the cells develop a more complex morphology (arrowheads). With FGF and PDGF cells maintain a bipolar morphology and migrate vigorously (bottom).

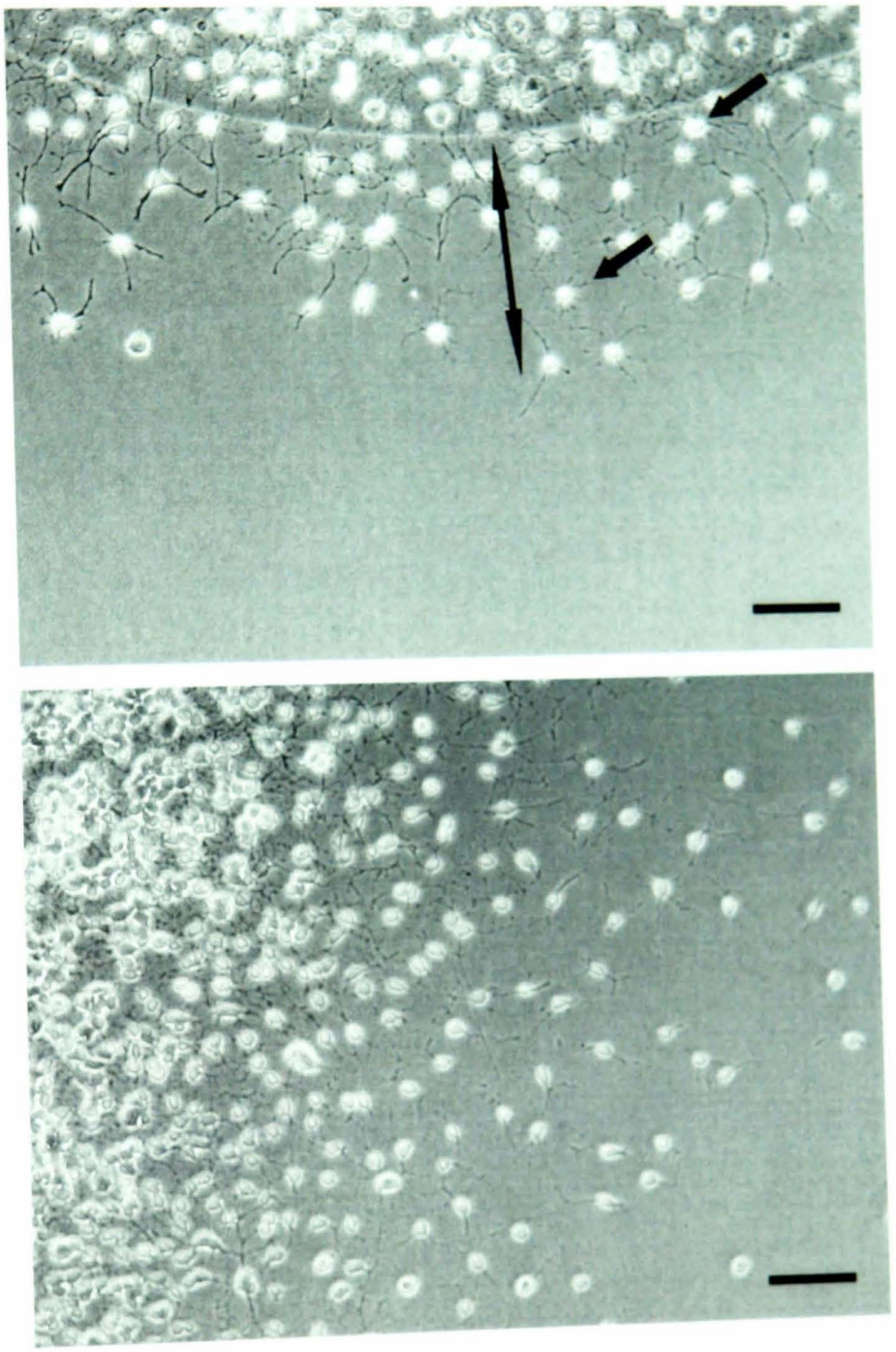
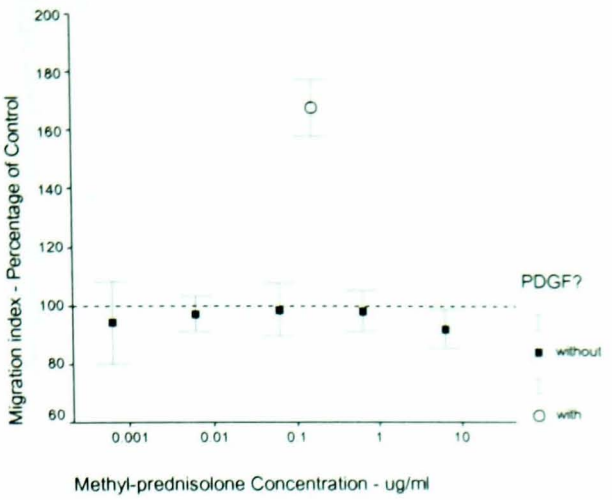
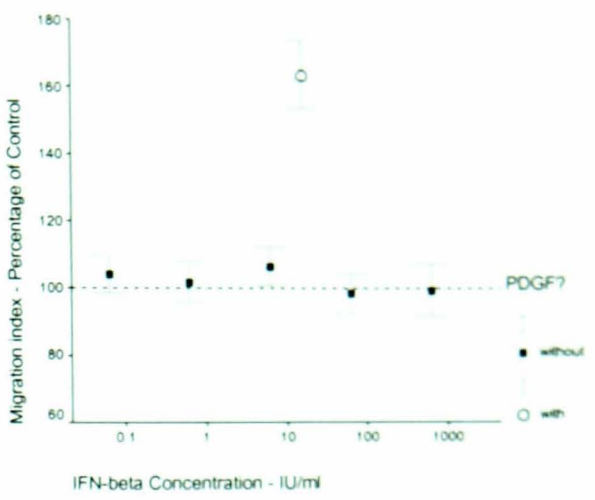


Figure 6-4 Individual dose-response curves for migration

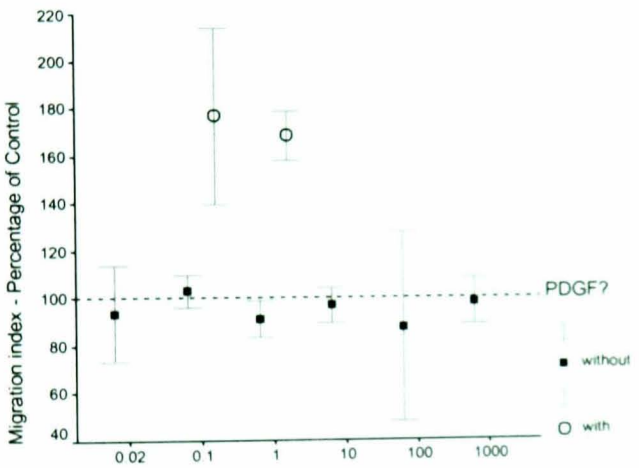
a. Methylprednisolone



b. Interferon-beta



c. Azathioprine



d. 6-Mercaptopurine

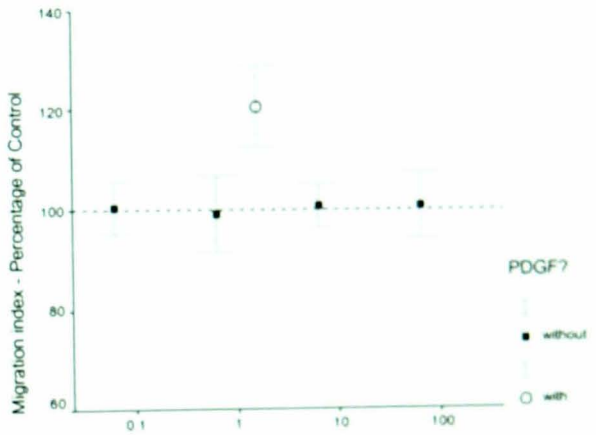
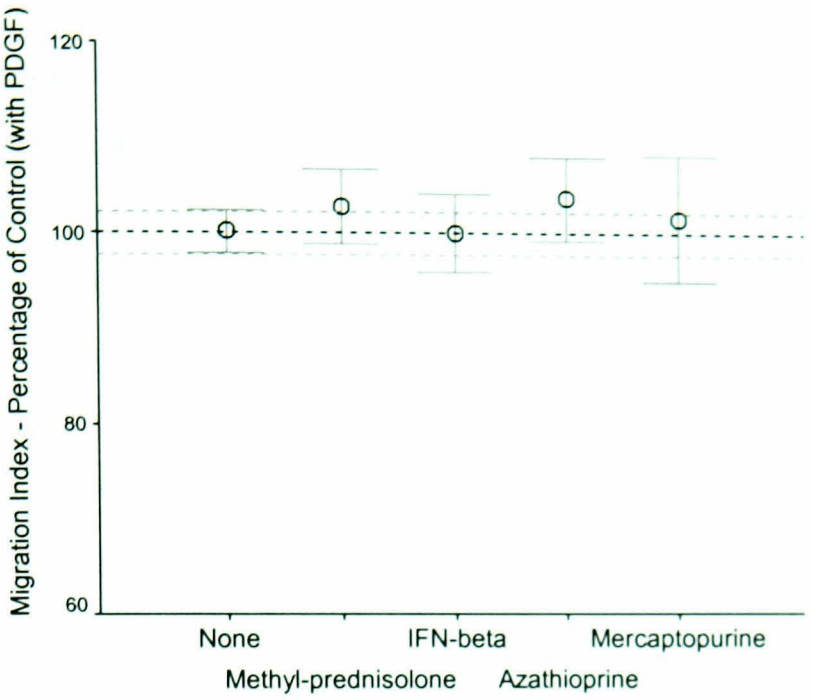


Figure 6-5 Progenitor cell migration under the influence of PDGF and FGF remained unaffected by anti-inflammatory drugs.



Chapter 7. Conclusion

The burden of disability caused by multiple sclerosis remains huge, and effective treatments elusive. In addition to the need for therapies that prevent myelin damage, there is a pressing need for repair strategies to alleviate existing neurological deficits. The underlying aetiology is poorly understood and the relationship between the different pathological components of the disease, myelin destruction, inflammation and progressive axon loss is complex. However, myelin damage dominates both the pathology and the pathophysiology of early disease, and we have argued that a second, important component of chronic disease, axonal damage, may be most convincingly attributed to persistent demyelination.

Remyelination is a striking example of regeneration in the brain, an organ otherwise notable for its failure to repair damage. However, while apparently effective both at restoring conduction (Smith et. al. 1979) and in preventing subsequent axonal degeneration (Kornek et al. 2000), intrinsic remyelination is limited, and ultimately fails to prevent the relentless progression of the disease. The cause of this failure remains a crucial question in designing treatment strategies, a question that depends for its solution on an understanding of the biology of the cell responsible; the adult human oligodendrocyte progenitor.

Much has been learnt about oligodendrocyte progenitors from studying experimental animals. An enormous amount is known about rodent oligodendrocyte progenitors, but there are sufficient differences between rodent and human cells to make the study of adult human oligodendrocyte progenitors essential. However, sources of these cells are scarce and, unlike rodent progenitors, it has not been possible to expand them *in vitro*, an uncomfortably pertinent example of the differences between the species. This problem of cell number has, par excellence, hampered the study of these important cells.

Notwithstanding these difficulties, some studies have succeeded in studying the adult human oligodendrocyte lineage. It is these very studies that have discerned their failure to proliferate in mitogens that expand rodent cells. However, they have also underlined the important similarities between the rodent and human oligodendrocyte progenitor, such as ganglioside expression and lineage potential *in vitro*. These similarities have underpinned the *a priori* hypothesis on which this work was based

namely that rodent and human cells are, despite some evidence to the contrary, biologically similar. It is this hypothesis which has informed our attempts to solve the problem of obtaining sufficient numbers of adult human oligodendrocyte progenitors to allow their study.

Chapter 2 described the result of one such attempt to use retroviral vectors carrying conditional oncogenes to transform primary adult human progenitors. Unfortunately, HW1 instead provides a salutary lesson about the risks of cross-contamination in cell culture.

Despite this particular example, the use of cell lines can and has provided dramatic insights into lineage biology. They can provide large numbers of cells in absolute homogeneity, and the judicious use of conditional oncogenes is a powerful tool in returning cells to an untransformed state. They can yield important insights into the biology of the parent cell, and properly manipulated cells can approximate more closely to the original than cells of a related species. Furthermore, they are frequently more faithful than cell lines arising stochastically in primary cultures, or grown from tumour tissue. However, all such studies require confirmatory experiments with primary cells, although it would be expected that these latter experiments could be performed with much smaller numbers.

In that the original reports were promising, it was eagerly anticipated that systematic assessment of HW1 would discern the crucial mitogen and media requirements that would then allow primary adult human oligodendrocyte progenitors to be expanded *in vitro*. The fact that the cells were overrun by a contaminating population does not negate this approach, which remains sound. However, it would always be a "stepping stone" to primary cell work for the reasons given. As such, the results of later chapters may render this approach, and the considerable time and resources required to repeat it, unnecessary.

Chapter 3 tested a different approach. Traditional assumptions of lineage potential are being increasingly challenged, and the idea of "reprogramming" cells to adopt a more primitive, and thus more proliferative, phenotype has some attractions. Ultimately, this type of approach suffers from a fundamental concern. To what extent do the observations of Kondo and Raff represent a tissue culture artefact? Do glia really reverse their differentiation pathway *in vivo*? This reversal of the developmental pathway in the laboratory may be retrograde in more than one sense if it affects the

phenotype of the progeny, and hence the conclusions of any experiment that uses them.

There are more provocative questions that arise from the discrepancies between the original work and the results of Chapter 3, despite the caveats discussed. If as we suggested, these discrepancies could be explained on the basis of different subpopulations of bipolar "O-2A" progenitors, then could some of these behave as a stem cell in their own right? The possibility that adult oligodendrocyte progenitors were in fact more akin to stem cell has been raised before (Noble et al. 1992) and their existence as a subpopulation of postnatal optic nerve "O-2A" progenitors is thus not inconceivable. If so, are adult human oligodendrocyte progenitors also stem cells? The results from Chapters 4 & 5 may help to answer these questions.

In Chapter 4 we returned to the traditional approach of addressing questions of cell biology to primary cells derived from the tissue of interest. While facing the substantial problem of limited cell numbers, this approach minimises the effects of manipulation *ex vivo*, and thus maximises the relevance of any findings. In addition, recent publications had provided useful suggestions for optimising the growth and isolation of these cells which fuelled optimism about the potential for their subsequent expansion *in vitro*.

An interesting early observation from these studies was the relative prevalence, in percentage terms, of the A2B5+GFAP- population in these cultures compared to previous observations (Roy et al. 1999;Scolding et al. 1995). This may reflect differential loss of other phenotypes, but growth factors were present in the culture media, so a more provocative possibility might be that these cells are proliferating prior to assessment. Evidence of cell proliferation was forthcoming during these experiments and we were able to demonstrate this by labelling PDGFαR+ cells in the final stages, so, once repeated, a major aim of this work will have been achieved. This will confirm that PDGF$_{AA}$ and FGF2, and in the last experiment NT-3, in combination with sufficient insulin to activate the IGF-receptor, are mitogenic for adult human oligodendrocyte progenitors. Nevertheless, the substantial expansion seen with rodent progenitors, while now eagerly anticipated, has yet to be demonstrated.

Secondly, we aimed to clarify the immunophenotype of these cells. Firstly, despite increasing use as a marker of oligodendrocyte progenitors in adult humans, the NG2 proteoglycan is also expressed by endothelial cells, fibroblasts, "type 2" and

occasional "type 1" astrocytes, as well as being expressed at low levels on some microglia. Secondly, provisional figures suggest NG2 antibodies fail to identify 68% of A2B5+/GFAP- cells in primary cultures. Both of these cast significant doubt on the rationale for using it as a marker of oligodendrocyte progenitors in adult humans. However, NG2 antibodies have identified a novel population of morphologically distinct, flattened cells with wide, clawed processes, which we have argued may be related to the oligodendrocyte progenitor despite a lack of A2B5 staining or later oligodendrocyte markers. Similar cells arise from an A2B5+ population and grow as clumps and colonies in FGF2 and $PDGF_{AA}$, implying significant proliferation. They are associated with, but distinct from A2B5+GFAP+ "type 2" astrocytes that also grow in these cultures and a major aim of future work is to identify the progeny of these intriguing NG2+ cells.

To do this requires a separation step, and the use of magnetic beads to this end was highly successful. The development of such a protocol allows some important questions to be addressed that cannot be asked of mixed cell cultures. The questions of NG2+A2B5-cell origin answered above exemplify the power of this approach. However it also provides a technique with which cells might be purified prior to transplantation, and its simplicity and efficacy could (and in certain haematological examples, has) been exploited in a clinical therapeutic setting.

Chapter 5 described the approach of harnessing the replicative potential of stem cells to overcome the problem of cell numbers. However, in contrast to Chapter 3, this approach made no attempt to divert cells from their normal maturation pathway. The first aim, to confirm the existence of stem cells in the adult human brain was broadly successful. Cells were noted to grow in suspension, expand under the influences of mitogens predicted to target stem cells, enlarge *in vitro* into spherical bodies with the appearance of neurospheres, and finally differentiate into cells of all three neural lineages. A particularly notable result was the demonstration of large numbers of cells bearing antigens specific for the oligodendrocyte lineage, substantial support for the assertion that this strategy could provide large numbers of mitogen-expanded oligodendrocyte lineage cells for research, and perhaps therapeutic purposes.

The neurosphere strategy produces colonies containing large numbers of cells that are best described as "expanded neural progenitors". This begs the question; can the oligodendrocyte progenitors be selected out and purified from these? A few small hurdles remain before the potential is fully realised. As discussed, the dissociation of

individual spheres requires some fine tuning to optimise the growth potential of the spheres. Progeny from these would then need to be sorted. The experience of magnetic bead separation, based on immunophenotypic insights also described in Chapter 4, provides an ideal method for this.

Two strategies in particular stand out as viable solutions to the problem of cell numbers; purification and direct expansion of adult human oligodendrocyte progenitors from primary cultures (Chapter 4) and primary expansion of adult human neural stem cells as spheres followed by dissociation and separation of developing oligodendrocyte progenitors (Chapter 5). However, does this work shed any light on how one might intervene therapeutically to augment remyelination in MS?

Therapeutic Remyelination in Multiple Sclerosis

A key question remains the root cause of the failure of intrinsic remyelination in MS. As has been discussed, many causes have been proposed for this, and different strategies could be devised to ameliorate each. Depletion of remyelinating cells is an explanation with considerable (Carroll et al. 1998;Duncan et al. 1997;Keirstead et al. 1998;Rosenbluth 1996;Scolding & Franklin 1999), but not universal (Penderis et al. 2003) support and this could be ameliorated by cell replacement strategies, such as that of therapeutic transplantation (Halfpenny et al. 2002), and this will be the focus of much of the subsequent discussion. However, an alternative approach to this problem, of relevance if impaired inward migration or late arrival of progenitors after axonal changes and/or glial scar formation was responsible, could be to find ways to manipulate the biology of the oligodendrocyte progenitors. In particular the functions of proliferation and migration, which are repeatedly associated with efficient remyelination, would be targets for the latter approach.

The hypothesis of dysregulation might, if correct, be the most complicated cause to redress, unless some orchestrating process could be identified that could itself be manipulated. The process of inflammation is an interesting contender. Long regarded as the villain in MS, its beneficial role is being increasingly appreciated. Remyelination occurs during the inflammatory phase of lesion formation (Smith & McDonald 1999;Babinski 1885). Indeed immunosuppressive agents have been associated with impaired remyelination (Smith & Franklin 2001), but it was not clear whether this was due to a direct effect on the remyelinating cells, or because of the

absence of stimulatory, chemokinetic or orchestrating signals from inflammatory cells. The fact that many of these agents are widely used to achieve short term gains in terms of resolving or suppressing acute relapses, adds some clinical relevance to these concerns. The study in Chapter 6 (also published in 2003 (Halfpenny & Scolding 2003)) goes some way towards focussing on inflammation *per se* as an important component of remyelination. It seems that future therapies may need to be more discriminatory about the way they alter the immune processes in MS.

While our understanding of the causes for failing remyelination progress, there is still a pressing need to design therapies to benefit those whose suffering will not wait for science to catch up. What if any prospect is there for treatment?

Cell replacement therapies remain a strong contender. A surprising variety of cells have been considered (Halfpenny et al. 2002), and it is worth reviewing the rationale behind some of these.

Adult Human Oligodendrocyte Progenitors as Therapeutic Remyelinating Cells.

Much experimental work on remyelination has focused on the oligodendrocyte lineage. There is an inherent logic in concentrating on these cells. They are the cells lost in multiple sclerosis; it is their normal function to myelinate the CNS, and spontaneous oligodendrocyte remyelination in multiple sclerosis bears witness to their substantial inherent capacity for remyelinating damaged areas of the brain. In addition, there is a wealth of experimental evidence to demonstrate the production of new myelin in experimental animals after transplantation of purified oligodendrocyte lineage cells (Duncan et al. 1992;Franklin & Blakemore 1997;Groves et al. 1993;Kocsis 1999;Rosenbluth 1996;Warrington et al. 1993) or cell lines (Barnett et al. 1993;Groves et al. 1993;Tontsch et al. 1994). There is also evidence to suggest that this process is accompanied by both improved conduction (Utzschneider et al. 1994) and demonstrable functional recovery (Jeffery et al. 1999).

The stage within the oligodendrocyte lineage most suitable for transplantation is important. Although some studies have suggested that mature differentiated oligodendrocytes are useful myelinating cells (Duncan et al. 1992), others suggest they have only limited capacity (Archer et al. 1997;Keirstead & Blakemore 1997). Comparative experiments have shown better myelin formation by implanted immature progenitors than mature oligodendrocytes (Warrington et al. 1993). The majority view is that mitotic potential is an important prerequisite for successful myelin formation

(Blakemore & Keirstead 1999), and that post-mitotic oligodendrocytes do not readily recapitulate their development to form mature myelin sheaths again.

A further significant advantage of the progenitor phenotype is its demonstrable migratory potential (Baron-Van Evercooren et al. 1996;Kiernan & Ffrench-Constant 1993;Warrington et al. 1993). Partly assisted by their bipolar morphology, these cells appear to migrate better through demyelinated lesions than their mature counterparts, which manifest a more complex morphology both *in vitro* and *in vivo*. However, endogenous progenitors only migrate 1-2mm to repopulate demyelinated areas (Franklin et al. 1997), indicating a very limited migratory potential through normal brain parenchyma. *In vitro,* astrocytes significantly impede progenitor migration (Duguid et al. 1985), as do mature oligodendrocytes (Jefferson et al. 1997), suggesting significant inhibition by surface molecules.

Transplantation studies also show poor survival and migration when progenitors are implanted into normal white matter, although they are able to populate and remyelinate when injected into, or very close to, lesioned tissue (Franklin et al. 1996). By contrast, these cells survive well in X-irradiated tissue, which depletes endogenous progenitor numbers (Hinks et al. 2001). Part of this increased survival may reflect competition between endogenous and implanted cells for survival factors, since progenitor numbers increase with increased availability of platelet derived growth factor (Barres et al. 1992) or glial growth factor 2 (Noel et al. 1999). The possibility of improving graft survival and proliferation by the use of growth factors has been explored *in vivo* with some success (Milward et al. 2000).

Investigations of human CNS glia have consistently demonstrated significant biological differences from rodent cells, so that data concerning rodent oligodendrocyte progenitors cannot be directly extrapolated to human glia. Early studies identified glia similar to the rodent OPC's in cultures derived from the fetal human CNS (Kennedy & Fok-Seang 1986). These cells can synthesise myelin in the dysmyelinated rodent CNS, even after cryopreservation (Seilhean et al. 1996). More recently, a progenitor was identified in cultures of adult human brain, and shown to possess similar immunophenotype and differentiation potential to its rodent counterpart (Scolding et al. 1995), but the response to growth factors appeared different. The work presented in Chapter 4 confirms the existence of this cell. However it suggests that the soluble mitogens for this stage of development may not be dissimilar to those of rodent cells under appropriate culture conditions (FGF2,

PDGFAA and IGF-stimulation +/-NT3), but further work is required to define the effects of individual mitogens in more detail. When mixed human oligodendrocyte lineage cells containing small numbers of progenitors were transplanted into demyelinated rat spinal cord, myelin membranes but not compact multi-lamellar myelin sheaths were observed (Targett et al. 1996). Methods for selection of these cells (for experimental purposes) from samples of human white matter have since been perfected (Chapter 4) (Roy et al. 1999;Windrem et al. 2002), but as yet myelin antigens but no demonstrable myelin sheath formation after transplantation of adult human progenitors have been published (Windrem et al. 2002).

With the demonstration of proliferative signals (Chapter 4), obtaining adequate numbers of oligodendrocyte progenitors for therapeutic purposes, whether autologous or heterologous becomes rather more feasible. The requirements for microbiological safety make the removal of serum from the cell harvesting process a minor obstacle; the demonstration of proliferation in serum-free medium has removed a rather more substantial barrier. It is anticipated that the adjustments required to transform demonstrable cell division to therapeutically viable expansion are now a realistic possibility, and are perhaps imminent. Further experiments using these cells are now required to determine whether mature, functional compact myelin can be achieved following transplantation into animal models of demyelination.

Adult Human Stem Cells as a Source of Therapeutic Remyelinating Cells

Stem cells themselves have potential as a source of remyelinating cells for transplantation therapies. As we have demonstrated here in Chapter 5, the adult human brain does contains neural stem cell and these can be expanded *in vitro*. Furthermore, they can give rise to significant numbers of oligodendrocyte lineage cells. Autologous cells could be harvested from the subventricular zone by endoscopic techniques through a small ventriculostomy, and re-implanted following *in vitro* expansion. Myelin formation following transplantation of apparently uncommitted stem cells from the adult human subventricular zone (albeit removed during tumour surgery) into the demyelinated rat spinal cord has been demonstrated (Akiyama et al. 2001b).

As our understanding of stem cells grows, and in particular of the cues that induce lineage commitment in them, alternative sources of autologous stem cell might become feasible. Dramatic recent examples of the generalised commitment of stem cells (Alison et al. 2000;Rietze et al. 2001), and in particular of the neural

differentiation of non-neural cells gives credence to this approach (Mezey et al. 2000b). Bone marrow derived cells are a particularly appealing candidate (Brazelton et al. 2000;Woodbury et al. 2000) because of their ease of retrieval and the considerable clinical experience of their use, and acutely isolated bone marrow fractions have been reported to remyelinate rodent spinal cord (Sasaki et al. 2001). These experiments also emphasise the importance of environmental influences to the process of lineage specification, the mechanisms of which remain predominantly conjectural.

Does this negate the requirement for lineage commitment prior to therapeutic transplantation? There are two important reasons why this is unlikely to be true. Firstly, while direct stem cell transplantation has been reported to yield myelin (Akiyama et al. 2001a;Sasaki et al. 2001), the myelin so formed was predominantly peripheral in type. Others report that commitment to the oligodendrocyte lineage is required for effective myelin formation (Smith & Blakemore 2000). While peripheral-type remyelination has been demonstrated to restore saltatory conduction (Honmou et al. 1996) and function (Akiyama et al. 2001a), there remain questions as to its trophic support of central axons. Secondly there is a more speculative concern. Stem cells are rare cells with an extraordinary specialist role. Their scarcity may not be without reason; their phenomenal replicative potential may carry an inherent risk of neoplastic transformation. Embryonic stem cell transplantation provides dramatic evidence of the tumorigenic potential of uncommitted stem cells. Furthermore, there is some conjecture that primary cerebral tumours may arise from just such immature cells and any increase in the population of these cells, through artificial means or otherwise, may lead to an increased risk of one of these cells accruing sufficient mutations for malignant transformation. One might propose that prior commitment, with an expected diminution of replicative potential, may increase the threshold for transformation and thus protect against this event.

Clinical Aspects of Transplantation Strategies

When to Intervene in the Disease Process?

Timing a remyelinating treatment in multiple sclerosis remains problematic. Clinically, the unpredictable course increases the temptation to defer a potentially hazardous intervention until progressive disability is established, and hope of spontaneous recovery extinguished - the first principal in any new therapeutic

endeavour must always remain "first, do no harm". Yet from a biological perspective, early intervention may offer significant advantages: as mentioned above, this is when spontaneous remyelination occurs, suggesting an optimally propitious environment, and whatever the specific reason(s) for failed endogenous repair, the majority of proffered explanations relate to chronicity (Charles et al 2002). Furthermore, the contribution of progressive axonal loss to secondary progressive multiple sclerosis (Bjartmar et al. 2000;Bjartmar & Trapp 2001) also mitigates against late intervention: little could be expected of repair strategies when the axonal framework for remyelination has been lost. In fact, as we have argued, axonal loss itself is a potent reason for augmenting remyelination early. Whatever the mechanisms responsible, remyelination appears, par excellence, to protect axons against later degeneration (Kornek et al. 2000). This may prove one of the most important reasons for therapeutic remyelination.

Thus the earlier the intervention, the greater the potential gain. But the significant obstacle to this approach is the risk of losing repaired areas, and carefully prepared and implanted remyelinating cells, to ongoing disease activity. Although advances in the area of MS immuno-therapy may give cause for cautious optimism, no current therapies are able to suppress myelin destruction completely. Concurrent use of potent immuno-suppressive agents, perhaps required in any case to prevent graft rejection, might help, yet there is evidence to suggest that some of these agents themselves (Smith & Franklin 2001), or suppression of inflammation in general (Edge et al. 1998;Platt 1996;Rosenbluth et al. 1993), may impair myelin repair. Chapter 6 would suggest that the onus of impaired remyelination in these cases may lie at the door of impaired inflammation, so considerable caution would be required if immunosuppressives were to be considered.

Where to Implant Cells in Disseminated Disease?

Most experimental studies have explored the effects of transplantation into a single site, but in multiple sclerosis almost innumerable areas of demyelination are commonly disseminated through the CNS. Clearly the prospect of multiple inoculations into widely disparate lesions is hardly realistic. What should not be overlooked, however, is that many plaques are clinically silent, while a disproportionate degree of disability frequently emanates from a few critical lesions in eloquent areas. Thus implantation into a very small number of carefully selected

lesions – for example, the optic nerves, the spinal cord, or the superior cerebellar peduncle – could yield a useful therapeutic dividend (Compston 1996). Early phase II clinical trials are likely to proceed on this basis.

An appealing solution to the problems of cell dispersal would be to encourage transplanted cells to migrate widely, as occurs during development (Kornack & Rakic 2001;Thomas et al. 2000;Yamaguchi et al. 2000). Migration through mature brain parenchyma is very limited, but pro-migratory agents have been identified, and supplementing cellular transplantation with growth factor infusions (Fricker-Gates et al. 2000), or even co-transplantation with growth factor secreting cells (Milward et al. 2000) has been tried with limited success. An alternative would be to block or suppress the expression or recognition of molecules that inhibit migration, an approach already under investigation to overcome inhibitors of axonal sprouting (Shearer & Fawcett 2001). Third, studies in neonatal dysmyelinating rodents have demonstrated significant cell dissemination after intraventricular transplantation (Learish et al. 1999) especially after two implantations separated by a few days (Mitome et al. 2001).

Assessing and Monitoring Transplantation Therapies

Considerable attention needs to be focussed both on clinical management and monitoring following transplantation. Three modes of treatment may potentially be required: ongoing exogenous trophic support of grafted cells, immune-suppression to prevent graft rejection, and adequate control of ongoing disease activity to reduce graft loss. These issues cannot be fully addressed prior to the early clinical trials, not least because of the substantial differences between animals and human models.

Regarding the first, little information is available, though the very limited number of animal studies do illustrate its potential impact (Milward et al. 2000). The adverse influence of anti-mitotic immunosuppressants on remyelination has been mentioned already. More positively, in multiple sclerosis, anti-rejection prophylaxis might help inhibit disease progression. The anti-leukocyte humanised monoclonal antibody Campath-1H, currently under investigation for the treatment of both (solid organ) transplant rejection and multiple sclerosis (Hale & Waldmann 1996;Moreau et al. 1996) is particularly promising in this respect. Nevertheless, if cell implantation is adopted, there may be a risk, despite immunosuppression, of inciting new anti-oligodendrocyte immune reactions, which not only see off the graft, but also augment underlying disease processes. Autografting might avoid this.

Sensitive and precise methods of monitoring graft survival, migration, efficacy of remyelination and early detection of uncontrolled growth will be essential if these therapeutic protocols are to be responsibly explored in a clinical setting. Furthermore, myelin repair without clinical improvement will be a hollow victory, so robust and reproducible methods of clinical assessment would need to be applied *ab initio*.

Non-invasive imaging is clearly attractive, most obviously magnetic resonance imaging (MRI) since it is widely available, safe and reasonably tolerated. Resolution is high, but contrast and specificity are more problematic. Standard protocols are unlikely to be sufficient to monitor remyelination, but considerable advances continue to offer new techniques, of which magnetisation transfer contrast is the strongest candidate for imaging remyelination (Barkhof 1997;Deloire-Grassin et al. 2000). Magnetic resonance spectroscopy (MRS) measuring N-acetyl aspartate (NAA) levels offer means of assessing any impact on local neuron/axon survival (Davie et al. 1995;De Stefano et al. 1995).

Using paramagnetic particles to label cells prior to transplantation, enabling their dispersion to be tracked by MRI (Bulte et al. 1999;Franklin et al. 1999;Lewin et al. 2000) has promise, though from a safety perspective, even the most trivial manipulation of cells prior to implantation would be better avoided. Furthermore, graft survival cannot be inferred from migration, since dead cells remain visible (Bulte et al. 1999), and this method not only fails to show new myelin formation but may also impair the ability of other MR modalities to do so. Positron emission tomography, although expensive and of limited availability, can be both sensitive, and specific, but no appropriate ligands are yet available for monitoring remyelination.

Serial neurophysiology may also prove valuable, and by monitoring conduction times may provide evidence of returning saltatory conduction in the targeted pathway(s). The optic nerve has particular advantages in this respect.

If the sites mentioned above – the optic nerve, spinal cord, or brainstem – are selected for the first experimental trials seeking clinical benefit, then programmes for rigorously monitoring both the biological and clinical effects of the intervention need to be established, including not only imaging and electrophysiological examination, but physical assessment of any clinically relevant effects. Specific clinical outcomes; measures of function, disability, and handicap must be adopted and tailored for each patient group. Ultimately, success will need to be measured using properly designed clinical trials, in which clinical outcomes are likely to wield the greatest weight.

Considerable advances in the field of clinical scale design have improved physical and functional measurement in multiple sclerosis (Hobart et al. 2000;Hobart et al. 2001b;Hobart et al. 2001a), so that the tools for assessing clinical outcome, on which remyelination therapies must stand or fall, are becoming available.

Final Comments

In conclusion, we have argued that there is a pressing need to augment myelin repair in Multiple Sclerosis, and to do so requires an understanding of the biology of the cell responsible; the adult human oligodendrocyte progenitor. The major obstacle to its study is the limited numbers of cells available, and its failure to proliferate *in vitro*. We have explored four separate approaches to this problem, and we contend that two offer a feasible solution. We have confirmed the presence of both stem cells and oligodendrocyte progenitors in cultures from adult human brain and have explored their immunophenotype and identified conditions for each that stimulate cell division *in vitro*. We have demonstrated how questions of oligodendrocyte biology can be answered, and how these answers may influence our understanding of the disease and its repair.

Publications

The following publications arose out of this work:-

Immune-modifying agents do not impair the survival, migration or proliferation of oligodendrocyte progenitors (CG-4) *in vitro*
Journal of Neuroimmunology 2003 June; 139(1-2): 9-16
C A Halfpenny, N J Scolding

Glial cells as Targets for Cytotoxic Immune Mediators Review Article
T M Benn, C A Halfpenny, N J Scolding
Glia. 2001 Nov; 36(2):200-11.

Cell Transplantation, Myelin Repair and Multiple Sclerosis Review Article
C A Halfpenny, T M Benn, N J Scolding
Lancet Neurology 2002 May; 1(1):31-40

Stem cells for the treatment of neurological disease Review Article
C M Rice, C A Halfpenny, N J Scolding
Transfusion Medicine 2003 Dec; 13(6): 351-361

The CG-4 cell line does not demonstrate the generalised potential of equivalent primary cells.
C.A.Halfpenny, N.J.Scolding Manuscript in preparation

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